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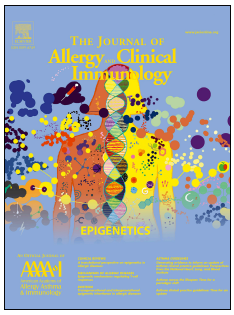

Vaccine against peanut allergy based on engineered Virus-Like-Particles displaying single major peanut allergens

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Title: Vaccine against peanut allergy based on engineered Virus-Like-Particles displaying single major peanut allergens

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Keywords: food allergy, novel therapy, Virus-Like Particles, safe vaccination

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ABSTRACT

Background:

Peanut allergy is a severe and increasingly frequent disease with high medical, psychosocial and economical burden for affected patients. A causal, safe and effective therapy is not available.

Objective:

We aimed to develop an immunogenic, protective and non-reactogenic vaccine candidate against peanut allergy based on Virus-like Particles (VLPs) coupled to single peanut allergens.

Methods:

To generate vaccine candidates, extracts of roasted peanut (Ara R) or the single allergens Ara h 1 or Ara h 2 were coupled to immunologically optimized Cucumber Mosaic Virus-derived VLPs (CuMVtt). BALB/c mice were sensitized intraperitoneally with peanut extract absorbed to Alum. Immunotherapy consisted of one single subcutaneous injection of CuMVtt coupled to Ara R, Ara h 1 or Ara h 2.

Results:

The vaccines CuMVtt-Ara R, CuMVtt-Ara h 1 and CuMVtt-Ara h 2 protected peanut sensitized mice against anaphylaxis after i.v. challenge with the whole peanut extract. Vaccines did not cause allergic reactions in sensitized mice. CuMVtt-Ara h 1 was able to induce specific IgG antibodies, diminished local reactions after skin-prick-tests and reduced the infiltration of the gastrointestinal tract by eosinophils and mast cells after oral challenge with peanut. The ability of CuMVtt-Ara h 1 to protect against challenge with the whole extract was mediated by IgG, as shown via passive IgG transfer. FcγRIIb was required for protection, indicating that immune-complexes with single allergens were able to block the allergic response against the whole extract, consisting of a complex allergen mixture.

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Conclusion:

Our data suggest that vaccination using single peanut allergens displayed on CuMVtt may represent a novel and safe therapy against peanut allergy.

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CAPSULE SUMMARY

Severe peanut allergy remains a critical pathology in clinical everyday life. In this preclinical study a vaccine based on Virus-Like-Particles displaying single major peanut allergens is presented as a possible safe and effective therapy against peanut allergy.

KEY MESSAGES

- Peanut allergy is a disease with increasing prevalence and avoidance of peanut is difficult to achieve
- Oral immunotherapy is effective, but time consuming and potentially dangerous for severely allergic patients
- An active vaccination based on engineered Virus-Like-Particles displaying single major peanut allergens generates protective IgG antibodies in a mouse model for peanut allergy
- Protection is FcγRIIb-dependent, showing the critical role of IgG-allergen immune complexes for protection against complex allergen mixtures

ABBREVIATIONES

VLPs: Viurs-like Particles
Ara R: roasted peanut extract
CuMVtt: Cucumber Mosaic Virus including tetanus toxin epitopes
PA: peanut allergy

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OIT: oral immunotherapy

SLIT: sublingual immunotherapy

EPIT: epicutaneous immunotherapy

TLR: Toll-like receptor

MCPT-1: mast cell protease-1

DARPin: designed ankyrin repeat proteins

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INTRODUCTION

Peanut allergy (PA) is a severe disease and is the most frequent cause of anaphylactic reactions and death among food allergies. The prevalence of PA in Western countries ranges between 1.4 to 3 % in children and is increasing (1). The disease typically develops early in life and only in about 20% of cases an outgrow of the allergy is observed (2). The economic and psychosocial consequences of PA are important (3). Strict avoidance of peanut is the most common strategy used by allergic patients, but is difficult to achieve. In terms of prevention, a randomized controlled trial in infants at high risk to develop peanut allergy found that early peanut consumption reduced the risk in comparison to peanut avoidance (4). In contrast, another randomized controlled clinical trial in normal risk infants found that early peanut exposure had no significantly protective effect on the development of peanut allergy (5). Finally, an observational trial in newborns again indicated that early peanut exposure had a protective effect (6).

In terms of therapy, several immunotherapy trials with peanut allergic patients have been performed, mainly using oral (OIT), sublingual (SLIT) and epicutaneous immunotherapy (EPIT) (7–9). These therapies showed beneficial effects on PA, but were associated with a long phase of desensitization (10). In addition, potentially dangerous systemic allergic reactions and disturbing gastrointestinal symptoms have been observed (11),(12). A recently published phase III clinical trial (13) investigating OIT showed positive results relating to desensitization against peanut, but patients with a history of severe anaphylaxis with bronchial asthma and chronic gastrointestinal symptoms were excluded in advance, similarly to previous trials (14). Thus, there is currently no causal, safe and ideally effective therapy of PA, especially for those patients with severe allergy.

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Peanuts contain a mixture of 12 allergens and numerous isoforms; considered as major allergens are Ara h 1 and Ara h 3 (members of the cupin superfamily) as well as Ara h 2 and Ara h 6 (members of the prolamin superfamily) (15) although IgE specificities vary among peanut allergic patients.

Most children with detectable peanut-specific IgE are not allergic to peanut (16). A previous study identified the central role of peanut specific IgG4 for clinical tolerance in sensitized but not allergic patients (17). The IgG4/IgE ratio to peanut was significantly greater in peanut sensitized but tolerant patients compared with that seen in allergic subjects, indicating that excess of IgG4 could contribute to clinical tolerance. Direct competition of IgG4 with IgE for the allergen and binding of IgG4-allergen complexes to the inhibitory receptor FcγRIIb on mast cells and basophils are supposed to be responsible for protection. These findings were consistent with observations in patients after successful peanut immunotherapy. These patients show an increase of specific IgG4 levels most notably to the major allergen Ara h 2, suggesting that Ara h 2 is an important allergenic protein in peanut (18).

Whether allergen immunotherapy works via rebalancing the T cell response or via induction of antibodies is a long standing debate (19). A protective effect against cat allergy through direct administration of two Fel d 1 specific IgG4 monoclonal antibodies has been demonstrated recently in a clinical study with cat allergic patients (20). IgG4 antibodies are thought to compete with IgE for Fel d 1 binding, thereby inhibiting the crosslinking of the FcεRI on mast cells and basophils. In addition, allergen-IgG immune complexes engage the inhibitory FcγRIIb, thereby blocking cellular activation. Based on these considerations, we postulate that a vaccine against peanut allergens able to induce a strong and specific IgG response may have the potential to protect peanut allergic patients.

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Virus-like particles (VLPs) are safe platforms for induction of protective antibodies and several VLP-based vaccines are commercially available including against Human Papilloma Virus and Hepatitis B Virus (21). In a previous study, a vaccine consisting of Qβ-derived VLPs coupled to the cat allergen Fel d 1 has been shown to be highly immunogenic and able to induce specific IgG antibodies in mice. Immunization of Fel d 1 sensitized mice with Qβ-Fel d 1 protected against anaphylaxis after challenge with Fel d 1 allergen (22). In a recent study, it has been shown that allergens displayed on Qβ-VLP are immunogenic but not reactogenic and fail to activate human mast cells (23). VLP could therefore constitute a platform to deliver allergens to peanut allergic patients in an immunogenic and effective but safe way.

In the current study, we aimed to develop and test vaccine candidates against peanut allergy based on the immunologically optimized VLP derived from Cucumber Mosaic Virus (CuMVtt) (24). In addition to the immunogenic properties of other VLPs including the repetitive three-dimensional scaffold (B cell activation) (25) and the RNA content (stimulation of TLR7 and TLR8), CuMVtt contains the a universal T cell epitope derived from the tetanus toxin (tt) genetically fused into the structure. The pre-existing T cell memory for tt is near universal in humans and incorporation of the tt epitope boosted T-cell response in randomly selected primary human T cells (24).

Here, we tested the efficacy of vaccine candidates composed of CuMVtt coupled to the whole extract of roasted peanut or to the single major allergens Ara h 1 or Ara h 2 in a mouse model of peanut allergy and demonstrate strong immunogenicity and ability to protect against local and systemic allergic reactions to allergen extracts. We finally delineate the mechanism of action of the vaccines.

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MATERIALS AND METHODS

Production of peanut extract, Ara h 1, Ara h 2

The extract of roasted peanut was obtained according to the protocol from Koppelmann et al. (26). Briefly, 10 g of roasted peanut kernels (roasted salted peanuts (Felix) produced by Intersnack, Poland) were manually ground with mortar and pestle in 20 mM Tris-HCl, 2 mM EDTA buffer (pH 8.2), the insoluble lipid part of peanut was removed through three low speed centrifugations (see supplementary figure S1 A).

Ara h 1 enrichment and purification from native peanut extract was performed with Ammonium sulfate fractionations, anion-exchange and size-exclusion chromatography (see supplementary figure S1 B, C).

Recombinant peanut antigen Ara h 2 was produced in E.coli C2566 cells transformed with pET42-Ara-h202-nhk. The biomass was sonicated in 10 ml buffer (20mM Tris/HCl pH 7.5, 1 mM EDTA, 0.1 mM PMSF, 0.1% TX-100, DNAse I (30 µg/10ml), RNAse (0.5 mg/10ml). Sonication of the cells was performed for 16 minutes (0.5 interval / 0.7 power, Hielscher UP200S). Additional 10 ml of the buffer were added, the suspension mixed on rotating mixer (10 rpm at room temperature for 1 hour). Inclusion bodies were collected by centrifugation at 11000 rpm for 30 min and washed with the same buffer. Next, insoluble Arah202-nhk pellets were solubilized in 10 ml of 6M guanidine-HCl containing 100 mM NaCl; 5 mM EDTA; 10 mM DTT; 20 mM Tris 8.5 and incubated at room temperature overnight. Then the sample was centrifuged (11000 rpm, 30 min) and slowly added to 110 ml refolding buffer (0.1M CAPS pH 9.5, 0.9M arginine, 0.3mM reduced and 0.03mM oxidized glutathione). After overnight incubation at room temperature the mixture was dialyzed against 200 volumes of buffer containing 20 mM Tris-HCl, 50 mM NaCl (ON, +4 °C) using SpectraPor dialysis membrane

(12-14 kDa). Refolded Arah202-nhk was diluted with 50 mM Tris-HCL (pH8.0), loaded onto Sepharose Q HP (XK16/20 column) and eluted with 1M NaCl in 50 mM Tris-HCl (pH 8.0). Arah202-nhk containing fractions were finally purified using Superdex 200 column (see supplementary figure S1 D).

Ara h 1 and Ara h 2 were identified by anti-Ara h 1 and anti-Ara h 2 polyclonal antibodies (Indoor Biotechnologies, Charlottesville, VA, USA) on Western blots followed by incubation with Horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG (Sigma-Aldrich, Staint-Loius, USA), see supplementary figure S1 B.

Production of CuMVtt

The production of CuMVtt was described in details in (24). Briefly, RNA from CuVMtt-infected lily leaves was isolated using TRI reagent (Sigma-Aldrich, Staint-Loius, USA). For cDNA synthesis, OneStep RT-PCR kit (Qiagen, Venlo, Netherlands) was used. The corresponding PCR products were cloned into the pTZ57R/T vector (Fermentas, Vilnius, Lithuania). After sequencing, the cDNA of CuMV coat protein (CP) gene was then subcloned into the NcoI/HindIII sites of the pET28a(+) expression vector (Novagen, San Diego, USA), resulting in the expression plasmid pETCuMVWT. The tetanus toxoid epitope coding sequence was introduced in CuMVWT gene, by two step PCR mutagenesis, resulting in expression vector pET-CuMVtt. For CuMVtt VLPs, E. coli C2566 cells (New England Biolabs, Ipswich, USA) were transformed with the CuMVtt CP gene-containing plasmid pETCuMVtt. The expression was induced with 0.2 mM Isopropyl-β-D-thiogalactopyranoside (IPTG). The resulting biomass was collected by low-speed centrifugation and was frozen at -20 °C. After thawing on ice, the cells were suspended in the buffer containing 50mM sodium citrate, 5mM sodium borate, 5mM EDTA, 5mM mercapto-ethanol (pH 9.0, buffer A) and were disrupted by ultrasonic treatment.

Insoluble proteins and cell debris were removed by centrifugation (13,000 rpm, 30 min at 5 °C, JA-30.50Ti rotor, Beckman, Palo Alto, USA). The soluble CuMVtt CP protein in clarified lysate was pelleted using saturated ammonium sulfate (1:1, vol/vol) overnight at 4°C. Soluble CuMV-CP-containing protein solution was separated from the cellular proteins by ultracentrifugation in a sucrose gradient (20–60% sucrose; ultracentrifugation at 25'000 rpm for 6 h at 5 °C (SW28 rotor, Beckman, Palo Alto, USA)). After dialysis of CMV containing gradient fractions, VLPs were concentrated using ultracentrifuge (TLA100.3 rotor, Beckman, Palo Alto, US; at 72,000 rpm 1 h, +5 oC) or by ultrafiltration using Amicon Ultra 15 (100 kDa; Merck Millipore, Cork, Ireland).

Generation of the vaccine CuMVtt-Ara R, CuMVtt-Ara h 1 and CuMVtt-Ara h 2

The peanut extracts, Ara h 1 or Ara h 2 were modified for the subsequent coupling to CuMVtt VLPs with SATA according to protocol of manufacturer (Thermo Fisher Scientific, Waltham, MA, USA). SATA reactions were performed for 30 min at 23 °C using 3.6 x molar excess of SATA for Ara R or 3.3 x for Arah1 and 10 x for Arah202-nhk. Unreacted SATA was removed by 4-times washing the proteins with 5 mM NaHPO4 Ph 7.5, 2mM EDTA using Amicon Ultra-0.5, 10 K filtration units (Merck-Millipore). Free Sulfhydryl groups in modified proteins were generated by deacetylation with 0.5 M Hydroxylamine (Thermo Fisher Scientific, Waltham, MA, USA), incubation for 2 hours at 23°C. The modified peanut extract, Ara h 1 or Ara h 2 were conjugated to CuMVtt using the cross-linker SMPH (Thermo Scientific, 10-molar excess, 60 min 23 °C). The coupling reactions were performed with 0.3 x molar excess of SATA modified Ara R, 0.3 x SATA-Arah1 or equal molar amount of Arah202-nhk regarding the CuMVtt (shaking at 23°C for 3 hours at 1200 rpm on DSG Titertek, Flow Laboratories). Unreacted SMPH and peanut proteins were removed using Amicon-Ultra 0.5, 100K (Merck-Millipore, Burlington,

MA, USA). VLP samples were centrifuged for 2 minutes at 14000 rpm for measurement on ND-1000.

Due to crosslinking of subunits, derivatization by SMPH leads to the characteristic ladder of CuMVtt monomers, dimer, trimers, tetramers, etc. The primary coupling band for CuMVtt-Ara h 1 appears as one CuMVtt monomer linked to one Ara h 1 protein at ~110 kDA. Coupling efficiency was calculated by densitometry (as previously described for IL17A-CMVTT vaccine (24)) with a result of ~ 20 to 30% meaning 60 peanut allergens molecules were linked to one particle.

Coupling confirmation and densitometry measurement were achieved on SDS-Page as shown in Figure 1A.

Mice experiments

BALB/c mice (Envigo, Huntingdon, UK) were purchased at the age of 6 weeks and kept at the DKF animal facility, Murtenstrasse 31, Bern. All animals were used for experimentation according to protocols approved by the Swiss Federal Veterinary Office (licence number BE 70/18).

To test the immunogenicity of the vaccines, 6-weeks-old naïve BALB/c mice were immunized s.c. either with CuMVtt coupled to Ara h 1 (30 µg CtMVtt-Ara h 1) or with Ara h 1 (10 µg). IgG levels 7 and 14 days after vaccination.

Six-weeks-old naïve BALB/c mice were sensitized to peanut by injecting twice i.p. with 5 µg roasted peanut extract mixed in 200 µl Alum (10 mg/ml Al(OH)3; Alhydrogel; InvivoGen, USA). For efficacy experiments, sensitized mice were vaccinated once s.c. either with 30 µg CuMVtt-Ara R, CuMVtt-Ara h 1 or CuMVtt-Ara h 2 in 200 µl PBS, 2 weeks after sensitization,

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control groups were injected with CuMVtt 30 μg. Challenge was performed i.v., via skin prick test or gavage.

For induction of anaphylaxis, sensitized mice were challenged i.v. with 20 μg roasted peanut extract in 200 μl PBS. Temperature was measured with a rectal probe thermometer (Vetronic Services LTD, Devon UK) before i.v. antigen challenge and monitored for 50 minutes after challenge. To assess physical fitness and activity after challenge, open field activity tests were performed starting 10 minutes after i.v. injection. Distance moved was recorded for 10 minutes for all group and evaluated with the video tracking system EthoVision XT-11 (Noldus Information Technology, Wageningen, Netherlands).

For passive vaccination, IgG antibodies were induced with CuMVtt-Ara h 1 immunization of naïve 6-weeks-old BALB/c mice. Pooled serum was collected and IgG isolated through Protein G sepharose column (GE Healthcare, Chicago, USA; according to manufactures instruction). Sensitized mice received once 150 μg of isolated IgGs in 200 μl PBS 24 hours before challenge, the control group 200 μl PBS. To assess the role of the inhibitory FcγRIIb receptor on basophils and mast cells on a systemic level, mice were injected i.v. with 150 μg anti-FcγRIIb antibodies (provided by Cragg M., Antibody and Vaccine Group, Southampton, United Kingdom) 24 hours before i.v. challenge with peanut extract. At the same time, as a control isotype antibody, 150 μg anti-Histidin antibodies were injected in CuMVtt-Ara h 1 vaccinated mice.

The local local allergic reaction was assessed by ear prick test. Mice were injected i.v. with 200 ml of Evans blue solution (0.5% in PBS). Afterword a drop of peanut extract solution (180 μg/20 μl PBS) was placed onto the outer ear skin of anesthetized mice. Pricks on the ear skin were performed with 23G (0.6 mm Å~25 mm) needles (Microlance; BD). To assess FcγRIIb receptor function, designed ankyrin repeat proteins (DARPins) (27) against mouse FcγRIIb receptor were

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used for blocking the receptor by means of local subcutaneous injection on the ears 10 minutes before the ear prick. Dye extravasations started immediately after antigen challenge. 40 min later, mice were sacrificed and ears were collected. Ears were collected, photographed with C300 device (Azure Biosystems Dublin, CA, USA) and surface of the blue extravasation was quantified by Fiji ImageJ software.

The infiltration of eosinophils in the gastrointestinal tract was assessed as follows: Sensitized and vaccinated mice were challenged for 3 days once per day with 100 mg roasted peanut or PBS via gavage. Mice were then sacrificed, stomach and proximal jejunum were collected, washed in PBS, fixed in paraformaldehyde 4% for 4 hours and embedded in paraffin. 5 µm sections were cut and stained with hematoxylin-eosin. 5 random fields per section (2 for stomach, 3 for jejunum) were examined with a Imager.M2 (Zeiss, Oberkochen, Germany) Microscope and scored for infiltration of eosinophils in the lamina propria according to a morphologic scale (1: no infiltration of eosinophils, 2: moderate infiltration of eosinophils, 3: strong infiltration of eosinophils).

For flow-cytometry analysis and eosinophils quantification, the first 10 cm of the small intestine were collected and mesenteric tissue was removed. The small intestine was longitudinally opened, washed in Hanks Balanced Salt Solution (HBSS) and cut in small pieces. Epithelial cells were removed through incubation for 20 min (37°C, on incubator shaker) in 35 ml pre-warmed HBSS containing 2% horse serum 0,005M EDTA (Sigma-Aldrich, Saint-Loius, USA) and 0.000308% DTT (Sigma-Aldrich, Saint-Loius, USA). Then intestinal pieces were washed in HBSS containing 2% horse serum and digested for 20 min in pre-warmed HBSS containing 2% horse serum, collagenase IV (50mg/100ml (Sigma-Aldrich, Saint-Loius, USA)) and DNase1 (2 mg/100 ml, (Roche Diagnostics, Switzerland)) solution (37°C, on incubator shaker). The

resulting suspension was filtered (40 μm pore) and centrifuged for 4 min at 370 g. The pellet was collected in HBSS containing 2% horse serum and DNase1 (2mg/100ml (Roche Diagnostics, Switzerland)). For eosinophils detection, cells were stained in HBSS on ice for 30 minutes: viability dye live/dead fixable blue dead cell stain kit (Invitrogen, Carlsbad, USA), CD45-BV711 (BioLegend, San Diego, USA), CD11b-PE (Becton Dickinson, Franklin Lakes, New Jersey, USA), Siglec F-PerCpCy5.5 (Becton Dickinson, Franklin Lakes, New Jersey, USA), GR1-AlexaF700 (BioLegend, San Diego, USA). Intestinal mast cells were stained with PE-Cy7-CD45 (BioLegend, San Diego, USA), APC-c-Kit (Becton Dickinson, Franklin Lakes, New Jersey, USA) and FITC-FcεRI (Thermo Fisher Scientific, Waltham, USA). Measurements were performed with FACS LSR II (Becton Dickinson, Franklin Lakes, New Jersey, USA) cytometer and analysis with FlowJo software (FlowJo LCC).

ELISA for determining peanut specific IgG

96-well Nunc MaxisorpTM ELISA plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 1 μg/ml in carbonate buffer at 4°C overnight. After blocking with PBS/0.15% Casein solution for 2 hours, plates were washed five times with PBS/0.05% Tween. Serial dilutions of sera were added to the plates and incubated for 2 hours at 4°C. Plates were then washed five times with PBS/0.05% Tween (PBST). Thereafter, HRP-labeled goat anti-mouse IgG (The Jackson Laboratory, Bar Harbor, ME, USA) antibodies were incubated at 4°C for 1 h. For determination of peanut extract specific IgG subclasses, biotin-labeled mouse anti-mouse IgG1 (The Jackson Laboratory, Bar Harbor, ME, USA), biotin-labeled mouse anti-mouse IgG2a (Becton Dickinson, Becton Dickinson, Franklin Lakes, New Jersey, USA) or biotin-labeled rat anti-mouse IgG2b (BioLegend, San Diego, USA) were used as detection antibodies for 1 hour at

4°C. Thereafter HRP-labelled streptavidin (DakoCytomation, Denmark) was incubated at 4°C for 1h. ELISAs were developed with TMB (3,30,5,50-tetramethyl- benzidine) and H2O2 and stopped with 1 mol/L sulfuric acid. Optical densities were measured at 450 nm. Half-maximal antibody titers are defined as the reciprocal of the dilution leading to half of the OD measured at saturation.

ELISA for determining mast cell protease-1 (MCPT-1)

MCPT-1 were measured in serum of mice collected one hour after i.v. challenge. The experiments were performed according to the manufactures instructions (MCPT-1 Mouse uncoated ELISA Kit, Invitrogen, Thermo Fisher, US).

***In vivo* Reactogenicity of CuMVtt vaccine**

Sensitized mice were challenged intravenously two weeks after completed sensitization with roasted peanut extract 20 µg or CuMVtt-Ara R 60 µg (corresponding dose of free and CuMVtt bound allergen). Anaphylaxis was assessed measuring temperature every 10 minutes for 50 minutes.To assess local reactogenicity of single major allergens, sensitized mice were challenged with Ara h 1 or CuMVtt-Ara h 1, in parallel with Ara h 2 and CuMVtt-Ara h 2 (with an allergen concentration of 0.3 mg/ml).

***In vitro* reactogenicity of CuMVtt vaccine, Basophils Activation Assay**

Experiments with blood of peanut allergic patients were approved by the local ethics Committee, KEK-Number 2018-00204. The experiments were performed according to the manufactures instruction (Flow CAST, Bühlmann, Switzerland). Briefly, whole blood of peanut allergic

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patients was incubated with free Ara h 2 or Ara h 2 bound to CuMVtt (same concentration related to the contained allergen). Basophils were detected with PE-fluorescence labelled anti-CCR3 monoclonal antibody. Cell activation was determined by FITC-fluorescence labelled anti-CD63 monoclonal antibody. Measurements were performed with FACS Canto (BD Biosciences) flow cytometer and analysis with Flowjow software (FlowJo LCC).

Simultanoeus binding and co-localization of IgE and IgG on basophils

Whole blood cells from naïve or peanut sensitized mice were incubated after lysing of the erythrocytes (Lysing buffer, Lonza, Walkersville, USA) with serum of naïve or CuMVtt-Ara h 1 immunized mice (1:5) together with peanut extract (1 µg/ml) in RPMI 164 for 30 min at room temperature. After washing, cells were stained with anti-mouse IgE-FITC (BD Becton Dickinson), anti-mouse CD49b-APC (BioLegend, San Diego, USA) and anti-mouse IgG-PE (Jackson ImmunoResearch, UK). Measurements were performed with FACS Canto (BD Biosciences) and analysis with FlowJo software (FlowJo LCC).

Imaging flow cytometry and analysis using Amnis IDEAS software

Imaging flow cytometry was performed using Image Streamx flow cytometer and the compatible INSPIRE system software (Amnis Corporation, Seattle, Wash). Cells were measured at 40× magnification and a flow speed flow coefficient below 0.2% indicating a stable core stream. Single cells were gated on the basis of “area” and “aspect ratio” features of the bright-field channel, which was set on channel 1. Focused cells were selected on the basis of “gradient root-mean-square” feature that measures the resolution of an image, whereby values above 60 were

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considered for further analysis. Basophils were gated based on APC anti-CD49b and FITC anti-IgE intensity. The data was analyzed using the IDEAS software (Amnis Corporation). IgE and IgG co-localization was assessed in IgG positive basophils using "bright detail similarity" features of the fluorescence emitted by FITC anti-IgE and PE anti-IgG in the co-localization wizard provided by the IDEAS software.

In vitro inhibition of mast cells activation

Murine bone marrow derived mast cells were cultured from BALB/c mice WT, as described in (28). Cells were sensitized against peanut by incubation with serum derived from peanut sensitized mice (ratio 1:10 in medium) overnight. After washing, mast cells were challenged with peanut extract (in a concentration of 1 µg/ml) preincubated with serum of naïve mice or with serum of CuMVtt-Ara h 1 vaccinated mice (ratio 1:10 in medium) for 30 minutes in the incubator (37 °C). After washing, cells were stained with anti-CD63-APC (BioLegend, San Diego, USA) to detect activation. Measurements were performed with FACS Canto (BD Biosciences) and analysis with FlowJo software (FlowJo LCC).

Statistical analysis

Statistical tests were performed with GraphPad PRISM 6.0 (Graph- Pad Software, Inc., La Jolla, CA, USA). Statistical significance is displayed as $P \leq .05$ (*), $P \leq .01$ (**), $P \leq .001$ (***), $P \leq .0001$ (****). Groups for IgG levels, dot surface after skin prick test, open field results (distance

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397 moved and velocity) and area under the curve were analyzed by unpaired two-tailed Student's t-
398 test. Anaphylaxis curves were analyzed by repeated measures two-way-Anova test.
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RESULTS

Coupling of roasted peanut extract, Ara h 1 or recombinant Ara h 2 to CuMVtt

To generate and test different vaccine-candidates against peanut allergy, we chemically coupled either the mixture of Ara h allergens contained in the extract of roasted peanut, or the purified single major allergen Ara h 1 or the recombinant Ara h 2 to the repetitive surface of CuMVtt, followed by removal of free allergen (supplementary Fig. S1 and S2) (24). Details for the allergen coupling are shown by SDS-PAGE in Fig. 1A. Densitometric analysis shows a coupling efficiency of about 20 to 30%. Therefore, approximately 15 to 20 µg of allergens are contained per 60 µg of vaccine. Fig. 1B shows with Ara h1 as an example that structure of VLPs is preserved after coupling.

Vaccination with CuMVtt-Ara R, CuMVtt-Ara h 1 and CuMVtt-Ara h 2 protects against anaphylaxis

To establish a mouse model for peanut allergy, BALB/c mice were sensitized i.p. at day 0 and day 7 with 5 µg roasted peanut extract absorbed to Alum. For induction of anaphylaxis, sensitized mice were challenged i.v. with different doses of roasted peanut extract in 200 µl PBS. Rectal temperature was assessed at the time point of injection and every 10 minutes for 50 minutes after challenge (Fig.1C). As shown in Fig. 1D the dose-dependent temperature drop as correlative parameter for anaphylaxis was assessed after allergen challenge. Mice receiving 100 µg peanut extract showed a temperature < 32 °C after 30 min and were euthanized.

To assess whether vaccinated animals were protected against anaphylaxis, BALB/c mice were vaccinated subcutaneously two weeks after sensitization with a single dose of 30 µg CuMVtt-

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Ara R, CuMVtt-Ara h 1 or CuMVtt-Ara h 2, or as a control CuMVtt alone (Fig. 2A). S.c. administration of CuMVtt alone or CuMVtt coupled to the allergens did not induce anaphylactic reactions in allergic mice, as shown by constant body temperature after injection (data not depicted). In contrast, s.c. injection with the corresponding amounts of free peanut allergens induced a significant anaphylactic reaction (supplementary figure S3).

Two weeks after vaccination, all groups were challenged i.v. with 20 µg roasted peanut extract and body temperature was monitored for 50 minutes (Fig. 2A). Groups immunized with CuMVtt alone showed a severe drop in body temperature. In contrast, CuMVtt-Ara R, CuMVtt-Ara h 1 and CuMVtt-Ara h 2 vaccinated mice were protected from anaphylactic reactions (Fig. 2B). An unexpected observation was the observed protection against the whole extract when mice were vaccinated against single allergens (Ara h 1 and Ara h 2) which allowed us to pursue new lines of investigation in order to gain better understanding and insights into the potential mechanism driving protection induced by the vaccine.

To determine the role of mast cells and IgE in the observed anaphylaxis, serum levels of mast cell protease 1 (MCPT-1) were measured by ELISA in an exemplary way for mice immunized against Ara h 1. Fig. 2C shows lower serum levels of mast cell protease 1 (MCPT-1) in the CuMVtt-Ara h 1 vaccinated group than in mice vaccinated with CuMVtt one hour after i.v. challenge. These data confirm the protective effect of the vaccine against anaphylaxis. Furthermore, increased serum MCPT-1 levels in the control group after challenge indicate mast cells degranulation and IgE-dependent induction of anaphylaxis in this mouse model (in line with (29)).

Displaying peanut extract on CuMVtt strongly reduces its reactogenicity

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Absence of reactogenicity of a vaccine against peanut allergy plays a fundamental role for clinical translation, since allergic reactions are feared and potentially dangerous. For this reason, severely allergic patients are usually excluded from clinical trials for immunotherapy based on allergens. To address the question of reactogenicity, BALB/c mice were sensitized with peanut extract and 2 weeks later challenged with 20 µg peanut extract or 60 µg CuMVtt-Ara R (corresponding to an equivalent amount of allergen present in the challenge). Temperature drop was extensive in the group challenged with free allergen (peanut extract), whereas animals challenged with peanut extract coupled to CuMVtt did not show altered temperature (Fig. 2D). Local reactogenicity was also monitored using skin prick test. In order to visualize extravasation, mice were pretreated with Evans Blue intravenously before the. Prick test. As shown in Fig. 2E, mice challenged with Ara h 1, Ara h 2 and Ara R developed a stronger allergic extravasation compared to mice challenged with CuMVtt-Ara h 1, CuMVtt-Ara h 2 and CuMVtt-Ara R respectively (allergen concentration of 0.3 mg/ml).

Next we performed *in vitro* basophil activation tests (BAT) with whole blood of peanut allergic donors. We compared expression of CD63, a marker for basophils degranulation, after whole blood incubation with Ara h 2 or CuMVtt-Ara h 2 in an equivalent concentration to the free allergen (30 ng/ml). Fig. 2F shows basophil activation upon free allergen stimulation; in contrast, incubation with Ara h 2 displayed on CuMVtt failed to activate basophils showing CD63 levels comparable to unstimulated blood. Collectively these results show that displaying peanut allergens on CuMVtt strongly reduces their reactogenicity. This observation is consistent with previous experiments performed with the cat allergen Fel d 1 in free form or coupled to Qβ-VLPs (23).

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Immunogenicity of CuMVtt-Ara h 1 in naïve and sensitized mice

Since Ara h 1 could easily be purified in large amounts from peanut extracts, CuMVtt-Ara h 1 was the preferred candidate from a production point of view and we focused subsequent experiments on this allergen as an exemplary model to explore this observed protection using a single allergen approach.

To this end, we addressed the immunogenicity of CuMVtt-Ara h 1 in a next step. Naïve BALB/c mice were immunized s.c. with 10 µg of Ara h 1 either coupled to CuMVtt or in free form. Peanut extract-specific serum IgG was measured 7 and 14 days after immunization. As seen in Figure 3A, peanut specific IgG titers were induced by Ara h 1 coupled to CuMVtt whereas strongly reduced titers were detected upon injection of free Ara h 1. Next, we investigated the immunogenicity of CuMVtt-Ara h 1 in mice previously sensitized with peanut extract by measuring IgG-subclass-titers at day 34 (one day before challenge). As shown in Fig. 3B vaccination with CuMVtt-Ara h 1 led to an increase in peanut extract-specific IgG1, IgG2a and IgG2b titers compared to basal levels of IgG subclasses in sensitized mice immunized with CuMVtt. These date indicate that immunization with CuMVtt vaccines has an impact on all IgG subclasses.

Passive vaccination with IgG generated with CuMVtt-Ara h 1 protects against anaphylaxis

To investigate the specific role of IgG antibodies in protection against anaphylaxis, BALB/c mice were injected with CuMVtt-Ara h 1 and IgG antibodies were purified from serum using protein A. Transfer of 150 µg of total IgG antibodies into peanut-sensitized mice protected from anaphylactic reactions. Protection was in a similar range as achieved by the vaccine itself, indicating that IgG antibodies were the major driver of vaccine efficacy (Fig. 3C).

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CuMVtt-Ara h 1 improves physical fitness of mice after i.v. challenge

Allergic mice challenged i.v. with peanut extract develop typical signs of systemic allergy including erected hairs and immobility in addition to hypothermia. In order to quantify fitness, we measured physical activity after challenge in an open field experiment. Effects of vaccination with CuMVtt-Ara h 1 on distances moved were recorded for 10 minutes, starting 10 minutes after intravenous peanut extract challenge. As shown in Fig. 4A, vaccination with CuMVtt-Ara h 1 resulted in significantly higher levels of distances the mice moved after challenge compared with the CuMVtt group, a finding consistent with the protection against temperature drop.

CuMVtt-Ara h 1 diminishes local mast cell degranulation in skin prick test

To examine the effect of vaccination with CuMVtt-Ara h 1 on local allergic reactions, skin prick tests were performed in peanut sensitized mice vaccinated with CuMVtt-Ara h 1 or CuMVtt as control. Allergen challenge by pricking into the ear skin with peanut extract induced vascular leakage in CuMVtt vaccinated mice. In contrast CuMVtt-Ara h 1 treated animals showed significantly smaller extravasation surface (Fig. 4B).

CuMVtt-Ara h 1 reduces infiltration by eosinophils and mast cells in the intestinal tract after oral challenge

Next, we wanted to address the protective capacity of the vaccine in a model of chronic food allergy. To this end, we measured the local inflammation in the gastrointestinal tract after gavage of peanut sensitized mice with ground peanut kernels in PBS. We quantified the infiltration of eosinophils in the lamina propria of stomach and proximal jejunum after oral challenge for 3

515 days. 5 random fields per section (2 for stomach, 3 for jejunum) were examined by microscopy
516 and scored for infiltration of eosinophils in the lamina propria according to a morphologic scale
517 (as described in (30), 1: no infiltration of eosinophils, 2: moderate infiltration of eosinophils, 3:
518 strong infiltration of eosinophils). As shown in Fig. 4C eosinophil infiltration in the
519 hematoxylin-eosin staining is reduced in mice immunized with CuMVtt Ara h 1 compared to
520 mice treated with CuMVtt alone.
521 For quantification of eosinophil and mast cells infiltration into the lamina propria of the proximal
522 small bowel folw cytometry analysis. To this end, 10 cms of the proximal jejunum were
523 collected, digested and single cell suspensions were stained for cell surface markers. Eosinophils
524 were defined as living $CD45^+$ $CD11b^+$ $SiglecF^{high}$ cells, mast cells were defined as living $CD45^+$
525 c-Kit^+ $Fc\varepsilon RI^+$ (gating strategy in Supplementary Fig. S3). Fig. 4D shows reduced eosinophil
526 infiltration (left panel) and mast cells infiltration (right panel) in the CuMVtt-Ara h 1 vaccinated
527 group which reached levels found in PBS challenged mice.
528

529 **The inhibitory FcγRIIb-receptor is required for protection induced by the single allergen-**
530 **vaccine CuMVtt-Ara h 1**
531 To investigate whether the inhibitory FcγRIIb-receptor present on mast cells and basophils is
532 involved in protection induced by IgG antibodies generated after CuMVtt-Ara h 1 vaccination,
533 peanut sensitized BALB/c mice were challenged i.v. 24 hours after injection of an anti-FcγRIIb
534 mononoclonal antibody (AT 128) to block FcγRIIb-receptor. As shown in Fig. 5A, protection
535 conferred by vaccination with CuMVtt-Ara h 1 was abrogated by systemic injection of FcγRIIb-
536 blocking antibodies. The protection was not affected when mice were injected with isotype
537 control IgG.

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538 The involvement of the inhibitory receptor FcγRIIb in protection was confirmed via skin prick
539 test after locally blocking the FcγRIIb-receptor with an inhibitor molecule (based on DARPin
540 technology) specific to FcγRIIb (31). As shown above, CuMVtt-Ara h 1 vaccinated mice
541 developed much smaller extravasation spots after ear skin prick test, but protection was
542 abrogated by local injection of FcγRIIb-blocking DARPin. Mice with blocked FcγRIIb show a
543 comparable leakage to unvaccinated challenged mice, demonstrating that inhibitory receptor
544 FcγRIIb is required for protection (Fig. 5B).
545
546 **Protection induced by vaccination is specific for the displayed allergen on CuMVtt**
547 The results so far indicate that vaccination against a single allergen protects against the whole
548 extract in a FcγRIIb-dependent manner. This implies that immune complexes made of IgG and
549 allergen are critical for protection. Hence, the vaccines should only work, if the respective
550 allergens are present in the challenge. To examine this question, we vaccinated peanut sensitized
551 mice with either CuMVtt or with CuMVtt-Ara h 1 or CuMVtt-Ara h 2 and challenged them in a
552 skin prick test with the extract, Ara h 1 or Ara h 2 (Fig 5C-E). As expected, mice challenged with
553 single allergens showed weaker reactions than the ones challenged with the whole extract (data
554 not shown). We therefore normalized vaccine induced protection to the reaction seen for the
555 respective allergens used for challenge. As shown in Fig. 5C and D protection was only observed
556 if mice were challenged with the allergen they were vaccinated against, but not with the other
557 allergen. In contrast, mice challenged with the extract containing all allergens were protected as
558 shown in Fig. 5 E.
559 These results indicate that protection induced by immunization with single allergens is not based
560 on cross-reactive antibodies but rather on the formation of immune complexes with the

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561 respective allergen used for immunization, causing engagement of the inhibitory FcγRIIb.

562

563 **IgE and IgG antibodies binds to basophils simultaneously and show co-localization on the**
564 **cell surface**

565 A requisite for the hypothesized mechanism of protection through the inhibitory FcγRIIb
566 receptor is the simultaneous binding of IgE and IgG-allergen-complexes on basophils and mast
567 cells. To examine this postulate, we incubated whole blood cells from naïve or peanut sensitized
568 mice with serum of naïve or CuMVtt-Ara h 1 immunized mice together with peanut extract and
569 analyzed IgG binding on basophils (CD49b and IgE positive cells were gated) by Flow
570 Cytometry and Imaging flow cytometry. As shown in Fig. 6A, basophils incubated with serum
571 derived from CuMVtt-Ara h 1 immunized mice and with peanut extract bind significantly more
572 IgG than basophils incubated with serum of naïve mice and with peanut extract. In other words,
573 IgG antibodies induced by the vaccine CuMVtt-Ara h 1 binds on basophils in presence of the
574 allergen. This effect is significantly increased in basophils derived from peanut sensitized mice
575 compared to naïve mice, indicating that presence of peanut specific IgE on the cell surfaces
576 increases binding of IgG-peanut extract complexes. Co-localization of IgE and IgG was highly
577 significant while there was no co-localization of IgG or IgE with CD49b which is a basophil
578 surface marker not related to antibody binding (Fig. 6C, D).

579

580 **Serum obtained from mice vaccinated with CuMVtt-Ara h 1 inhibits mast cells activation**

581 To confirm *in vitro* the inhibitory effect of vaccination with CuMVtt-Ara h 1 on mast cells
582 activation, bone marrow derived mast cells were first sensitized with serum obtained from
583 allergic mice. After washing, the challenge was performed with peanut extract preincubated with

584 serum from mice vaccinated with CuMVtt-Ara h 1 or CuMVtt-immunized mice as a control.
585 As shown in Fig. 6E mast cell activation was significantly inhibited by the presence of serum
586 from CuMVtt-Ara h 1 mice confirming the inhibitory effect of IgG-allergen complexes.
587

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DISCUSSION

This study uses a preclinical setting to test vaccine candidates for PA. The vaccines are based on the immunologically optimized plant VLPs CuMVtt coupled either to peanut extract or to the single major allergens Ara h 1 or Ara h 2. Vaccination against either Ara h 1 or Ara h 2 was sufficient to induce protection against the whole peanut extract consisting of multiple allergens, as assessed in an anaphylaxis model, by skin prick testing and small bowel eosinophils and mast cells infiltration after gavage.

Efficacy of systemic immunotherapy is thought by some to rely on induction of allergen-specific regulatory T-cells (32) or a shift from Th2 cells toward Th1 cells (33) with a consecutive decrease of allergen specific IgE. Induction of humoral responses with generation of allergen specific IgG during immunotherapy (increasing the ratio IgG/IgE) is discussed by others as an essential element responsible for induction of allergen-tolerance (34),(35),(36). In this model, IgG is supposed to both compete with IgE for the allergen preventing crosslinking of the FcεRI receptor as well as engaging FcγRIIb. A strong “proof of principle” in humans for the protective effect of allergen specific IgG was obtained through administration of two monoclonal Fel d 1 specific IgG antibodies in cat allergic patients, showing significantly improved symptoms after nasal stimulation tests in a placebo controlled trial (20). Therefore, a sufficient high titer of IgG antibodies with adequate affinity/avidity for the allergen is able to diminish allergic symptoms after exposure. In our study, we found a significant increase of specific IgG responses after CuMVtt-Ara h 1 immunization and demonstrate that transfer of purified IgG fractions could confer protection against allergic reactions. This supports the role of IgG in the mechanism of protection induced by the vaccine candidates tested here. Moreover we could show that IgG was

not anaphylactogenic itself as transfer of IgG from immunized mice did not induce reactogenicity. This is most likely because high levels of allergens are required for IgG to induce anaphylactic reactions. A limitation of this study is that some experiments were done by way of intravenous challenge, which is not physiologic for peanut exposure in humans. Nevertheless as previously shown (22), parenteral injection of allergen may indeed represent a model for systemic exposure, allowing investigations of vaccine induced protection against systemic symptoms and related mechanisms.

We have shown in this study that vaccination against single allergens results in protection against peanut caused by a complex allergen mixture. Protection was transferrable by IgG antibodies and the inhibitory receptor FcγRIIb present on mast cells and basophils was critical for reduced allergic symptoms. Fig. 7 shows a model of the proposed mechanism of action. In allergic patients, peanut allergens engage IgE molecules on mast cells and basophils, causing their activation and the allergic response (Fig. 7, left part). In presence of high levels of IgG antibodies specific for a single allergen, IgG-immune complexes will be formed and bind FcγRIIb, causing inhibition of all IgE-mediated signals, including those from IgE molecules cross-linked by other allergens (Fig. 7, right part). This explains why IgG antibodies against single allergens are able to block cellular activation by whole allergen-extracts. These results are in line with previous studies showing that FcγRIIb was able to inhibit signals generated by activating receptors that were sensitized with non-cross-reacting IgE and were not directly co-engaged with FcγRIIb (37).

Patient`s IgE specificities for peanut allergens and even corresponding epitopes can be determined in most cases. This knowledge will enable to generate a patient specific vaccine against the most abundant allergen with high IgE but low IgG responses. Potential cross

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reactivity may also be taken into account, since different peanut allergens are known to be cross-reactive (38),(39). Hence, some protection against additional allergens may also be caused by cross-reactivity and regular (cross-reactive) allergen-neutralization.

In summary this study delivers a strong preclinical package for a vaccine using a single-peanut-allergen approach displayed on CuMVtt. It combines an excellent safety profile (absence of allergic reactions induced by the vaccine) with an equally attractive efficacy profile as vaccination strongly reduces systemic and local allergic symptoms upon challenge with the whole allergen extract. Moreover, using our vaccine approach the fact that immunizing against one single allergen protects against an allergen mixture is striking and could be applied in different relevant allergies, frequently caused by sensitization against more than one allergen. In addition PA is an increasing and severe disease, not comparable with many other allergies, hence we would like to highlight the translational potential of this study.

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CONFLICTS OF INTEREST

M.F.B. and T.M.K. declare to be involved in a number of companies developing VLP-based vaccines. M.D.H., M.F.K., M.A.S. are employee of Allergy Therapeutics Ltd. (United Kingdom). The other authors declare no further conflict of interests.

AUTHOR CONTRIBUTIONS

F.S., A.Z., I.B., L.Z., E.R., P.E., L.M. and D. vW., T.G., C.M. and M.M. performed experiments and interpreted data. F.S. M.D.H., M.F.K., M.A.S., T.M.K and M.F.B. designed the study. F.S. M.D.H., M.F.K., M.A.S., T.M.K and M.F.B wrote the manuscript.

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FIGURE LEGENDS

Figure 1.

Vaccine generation and establishment of a mouse model of peanut allergy.

A) Analysis of peanut extract, Ara h 1, Ara h 2 and coupling reactions with CuMVtt by SDS Page 4-12% gradient. Coupling bands show successful reaction. B) Electron microscopy image of CuMVtt coupled to Ara h 1. Vaccine particles are morphologically not aggregated (for dynamic light scattering analysis see supplementary Figure S2). C) Experimental design for establishment of peanut allergy mouse model. 6-weeks-old naïve BALB/c mice were injected i.p. with 5 µg roasted peanut extract mixed in 200 µl Alum at day 0 and day 7. Challenge with roasted peanut extract was performed at day 21. D) Temperature after challenge was measured rectally every 10 minutes for 50 minutes. Dose-dependent anaphylaxis corresponding to temperature drop after challenge with roasted peanut extract, means +/- SEM are shown (n = 3 mice per group). Data are representative of two independent experiments. Mice showing temperature < 32°C were euthanized (challenge with 100 µg roasted peanut extract) according to regulatory protocols. Anaphylaxis curves were analyzed by repeated measures two-way-Anova test, comparing the PBS challenged group to peanut extract challenged groups (dose dependent anaphylaxis).

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Figure 2.

Vaccine CuMVtt-Ara h 1, CuMVtt-Ara h 2 and CuMVtt-Ara R protect against anaphylaxis in a mouse model of peanut allergy.

A) To assess efficacy of generated vaccine mice were sensitized with i.p. injection of 5 µg roasted peanut extract mixed in 200 µl Alum at day 0 and day 7. Mice were vaccinated with 30 µg of CuMVtt-Ara h 1, CuMVtt-Ara h 2 or CuMVtt-Ara R on day 21. Challenge was performed on day 35 with 20 µg roasted peanut extract. B) Temperature after challenge to assess anaphylaxis was measured rectally every 10 minutes for 50 minutes. Left panel shows temperature course after challenge. Means +/- SEM are shown, (n = 4 to 5 mice per group). Data are representative of 3 independent experiments. Statistical significance was analyzed by two-way-Anova test. Right panel shows statistical analysis performed with unpaired t-test of related area under the curve (depicted +/- SEM). C) To assess the role of mast cells after challenge in CuMVtt vaccinated mice compared to CuMVtt-Ara h1 vaccinated mice serum MCPT-1 levels were measured in ELISA. Data are representative of two independent experiments (depicted +/- SEM).

D) Displaying allergens on CuMVtt reduces its reactogenicity. To show this, BALB/c mice were sensitized with peanut extract and 2 weeks later challenged i.v. with 20 µg peanut extract or 60 µg CuMVtt-Ara R (corresponding amount of allergen present in the challenge). To assess local reactogenicity, sensitized mice were challenged with Ara h 1 or CuMVtt-Ara h 1, in parallel with Ara h 2 and CuMVtt-Ara h 2 (with an allergen concentration of 0.3 mg/ml). Mean are shown +/- SEM (n = 3 mice per group), the graph shows results representative of three independent experiments. F) Ara h 2 was able to activate *in vitro* human basophils of peanut allergic patients (n = 3) in a basophil activation test (BAT, activated basophils defined as CCR3 +, CD63 + cells

892 by flow cytometry), whereas Ara h 2 coupled to CuMVtt did not activate basophils compared to
893 unstimulated cells. The experiment was performed once per patient, depicted results are
894 representative for all 3 patients.
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Figure 3.

Immunogenicity of CuMV-Ara h 1.

A) BALB/c mice were injected s.c. either with a total Ara h 1 amount of 10 μg in free form or coupled to CuMV, left panel. Serum anti-roasted peanut extract IgG levels were measured 7 days and 14 days post injections. Shown are means +/- SEM (n = 3 mice per group). Data are representative of 2 independent experiments. Peanut sensitized mice were immunized with 30 μg CuMVtt or CuMV-Ara h 1 s.c., serum anti roasted peanut extract total IgG were measured at day 34 (one day before challenge), right panel, shown are means +/- SEM (n = 5 mice per group). Data are representative of 2 independent experiments. B) IgG-subclasses specific for roasted peanut extract were measured at day 34 (one day before challenge), vaccination with CuMVtt-Ara h 1 led to an increase of OD50 titers of IgG1, IgG2a and IgG2b titers. Means +/- SEM are shown (n = 4 to 5 mice per group). Data are representative of 3 independent experiments, statistical analysis was performed with multiple t-test for corresponding dilutions. C) IgG antibodies were induced with CuMVtt-Ara h 1 immunization of naïve 6-weeks-old BALB/c mice. Pooled serum was collected from naïve or CuMVtt-Ara h 1 vaccinated mice and IgG-antibodies were isolated through Protein G sepharose column. Sensitized mice received once 150 μg of isolated IgGs from vaccinated mice in 200 μl PBS i.v. 24 hours before challenge, the control group 150 μg of isolated IgG from naïve mice in 200 μl PBS i.v.. Challenge was performed with an i.v. injection of 20 μg roasted peanut extract. Means +/- SEM are shown (n = 5 mice per group).

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Figure 4.

Effects of CuMVtt-Ara h 1 vaccine on physical fitness, skin prick test and on eosinophils infiltration in the proximal small bowel after challenge with roasted peanut extract in a mouse model of peanut allergy.

A) Peanut sensitized BALB/c mice were challenged i.v. with 20 μg roasted peanut extract, total distance moved was measured starting 10 min after i.v. injection for 10 minutes in an open field experiment. CuMVtt-Ara h 1 increased distance the mice moved after challenge. Mean are shown +/- SEM. (n = 9 mice for no challenge group, n = 12 for CuMVtt and CuMVtt-Ara h 1 group). Data are generated with 3 independent experiments. Heatmap plots shows representative movements of one mouse during 10 minutes of for each group. Statistical analysis of distance moved is performed with unpaired t-test. B) Sensitized and vaccinated mice were injected i.v. with 200 ml of Evans blue solution, prick test was performed with peanut extract on the ear skin under anesthesia. Surface of the extravasation was quantified using Fiji ImageJ software Means +/- SEM are shown (n = 3 mice per group). Data are representative of 2 independent experiments. Statistical significance was analyzed by unpaired t-test. C) and D) Sensitized and vaccinated mice were challenged for 3 days once per day with 100 mg roasted peanut or PBS via gavage. Mice were then sacrificed, stomach and proximal jejunum was collected. Hematoxylin-Eosin staining (C, arrows: example of eosinophil) and FACS analysis (D) show reduced eosinophils infiltration (left panel) and mast cells infiltration (right panel) in the lamina propria of CuMVtt-Ara h 1 vaccinated mice compared to the CuMVtt group. Mean are shown +/- SEM, data are representative of 2 independent experiments for hematoxylin-eosin sections, (n = 5 mice per group); FACS analysis regarding the proximal jejunum shows results of one experiment (n = 5 per group for CuMVtt and CuMVtt-Ara h 1 group, n = 3 for PBS challenge).

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Figure 5.

FcγRIIb is required for protection.

A) To assess systemic FcγRIIb receptor function, 150 µg of anti-FcγRIIb monoclonal antibody (AT 128) were administered i.v. 24 hours before allergen challenge, the control group received 150 µg of isotype IgG. Depicted statistical analysis shows difference between CuMVtt-Ara h 1 + anti-FcγRIIb and CuMVtt-Ara h 1 + isotype IgG control antibodies (n = 6 mice per group). B) To assess local FcγRIIb receptor function, a DARPin molecule against FcγRIIb receptor was used for blocking the receptor by means of local subcutaneous injection on the ears 10 minutes before the ear prick.

C), D), E) Protection induced by vaccination is specific for the displayed allergen on CuMVtt. Sensitized BALB/c mice were vaccinated against Ara h 1 or Ara h 2 and challenged in a skin prick test with the whole extract, Ara h 1 or Ara h2. C) Peanut sensitized mice vaccinated with CuMVtt-Ara h 1 and challenged with Ara h 1 were protected. In contrast vaccination with CuMV-tt-Ara h 2 failed to induce protection after challenge with Ara h 1. D) In parallel vaccination with CuMVtt-Ara h 2 protected in case of challenge with Ara h 2, but vaccination with CuMV-Ara h 1 failed to protect mice after challenge with Ara h 2. E) CuMVtt-Ara h 1 and CuMV-Ara h 2 both protect in case of challenge with the whole peanut extract in peanut sensitized mice In C-E the vaccine induced protection is shown in % of change to the reference value obtained for the respective allergen used in the challenge and defined as 100%. Mean are shown +/- SEM (n = 3 mice per group). Data are representative of 2 independent experiments. Statistical analysis is performed with t-test.

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Figure 6.

IgE and IgG antibodies bind to basophils simultaneously and show co-localization on the cell surface.

A) To examine binding of IgE and IgG on basophils, whole blood cells from naïve or peanut sensitized mice were incubated with serum of naïve or CuMVtt-Ara h 1 immunized mice together with peanut extract. IgG binding on basophils (CD49b and IgE positive cells were gated) was quantified by Flow Cytometry (A) and Imaging flow cytometry (B, C, D). Depicted data (A) were obtained in 3 independent experiments, +/- SEM. B), C), D) Co-localization of IgE and IgG was assessed in IgG positive basophils of sensitized mice incubated with serum of mice immunized with CuMVtt-Ara h 1 and peanut extract. Data are shown +/- SEM (n=3 mice per group). E) Murine bone marrow derived mast cells were sensitized with serum of peanut sensitized mice. Mast cells were challenged with peanut extract incubated with serum from naïve or CuMVtt-Ara h 1 vaccinated mice. Activation of mast cells was quantified by Flow Cytometry gating CD63 positive cells. Data are representative for 3 independent experiments.

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Figure 7.

Protection induced by CuMVtt-Ara h 1 vaccine against peanut allergy, proposed mechanism of action by which generated anti-Ara h 1 IgG antibodies stimulate the inhibitory receptor FcγRIIb on mast cells and basophils. FcεRI-meditated degranulation. ITAM, Immunoreceptor tyrosine-based activation motif. ITIM, immunoreceptor tyrosine-based inhibitory motif. SHIP, Src homology domain 2– containing inositol phosphatase. Syk, spleen tyrosine kinase. Scheme adapted from *(40)*.

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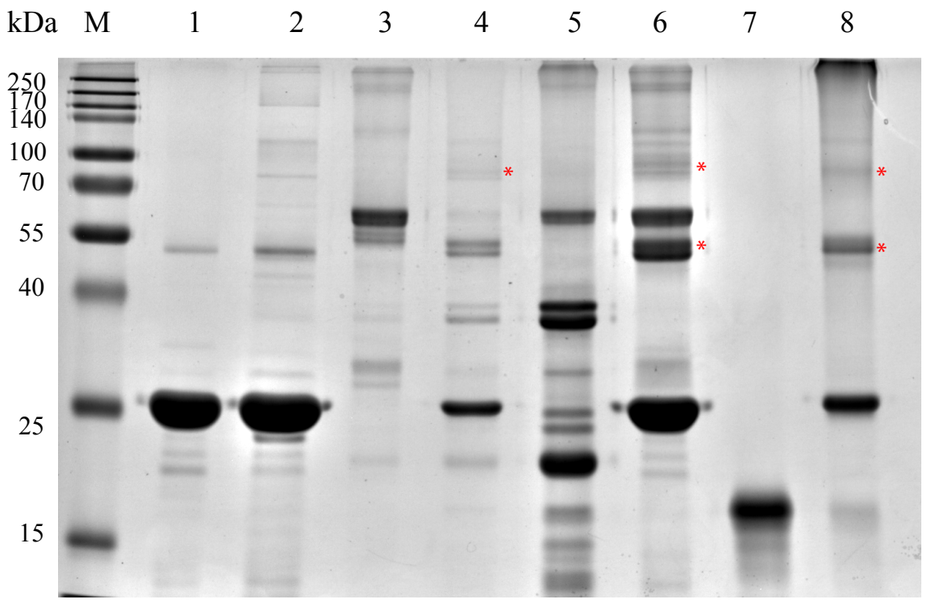
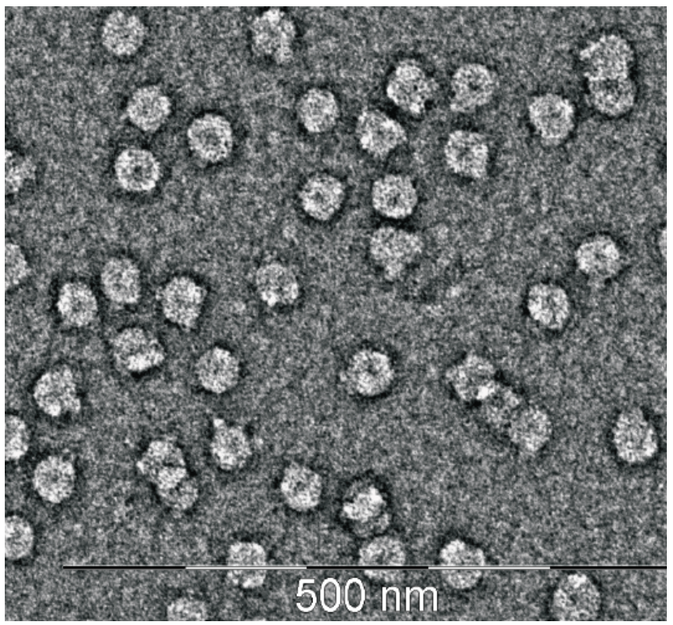
Figure 1

A



M - Marker PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa
1 - CuMVtt
2 - CuMVtt (2.5xSMPH)
3 - Ara h 1
4 - CuMVtt (2.5xSMPH)+0.3xArah1 (4xSATA)
5 - Ara R
6 - CuMVtt (2.5xSMPH)+0.3xAraR (4xSATA)
7 - Ara h 2
8 - CuMVtt (2.5xSMPH)+1xArah2 (4xSATA)
* - coupling band

B



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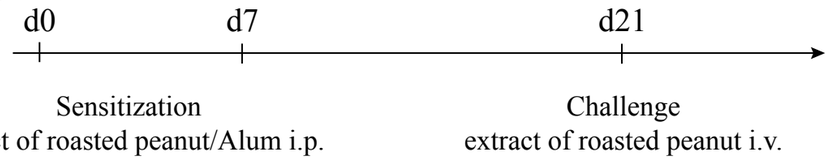

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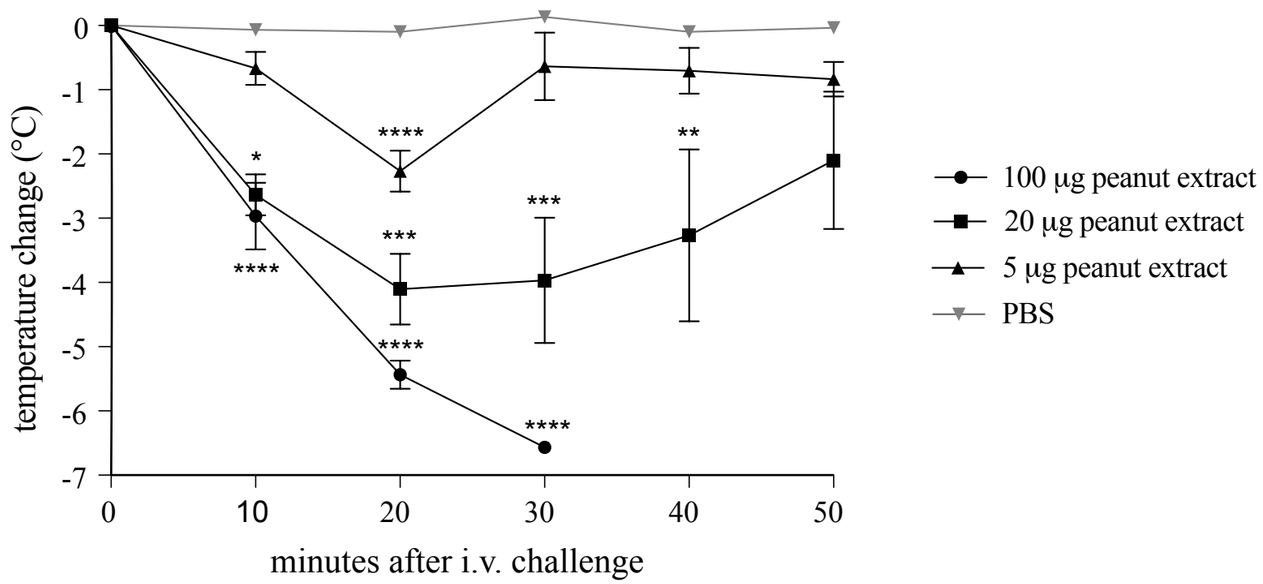

Figure 2

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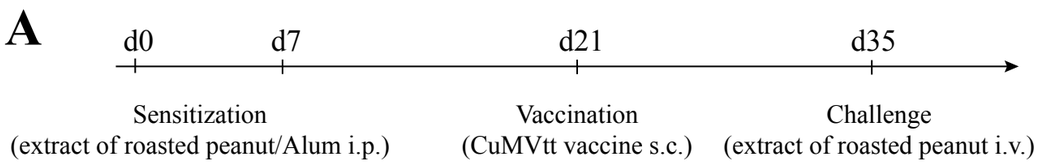

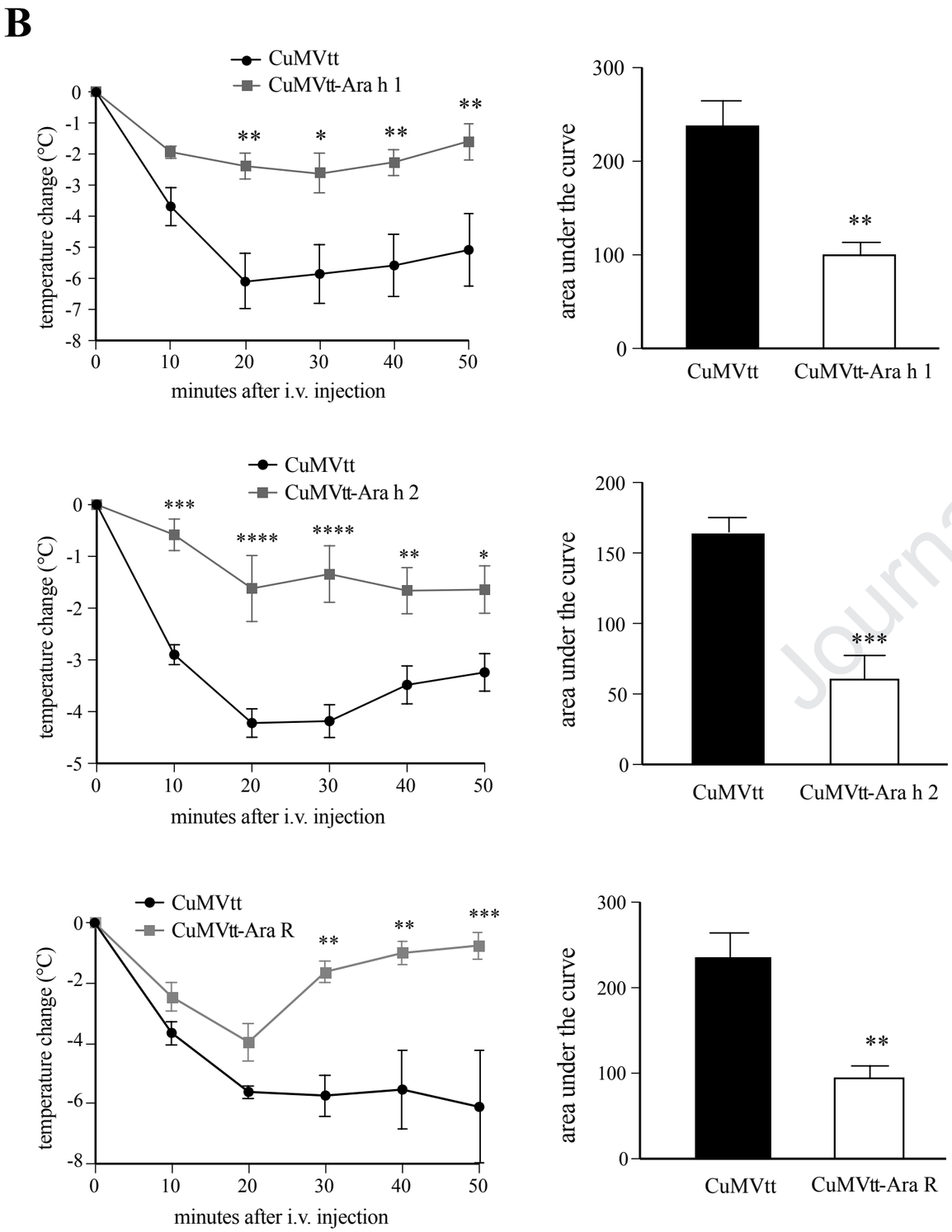

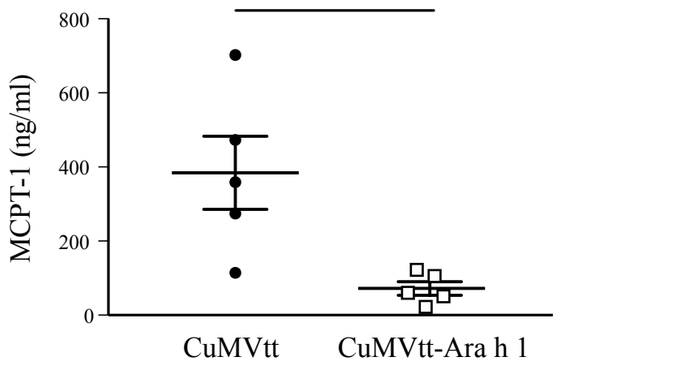

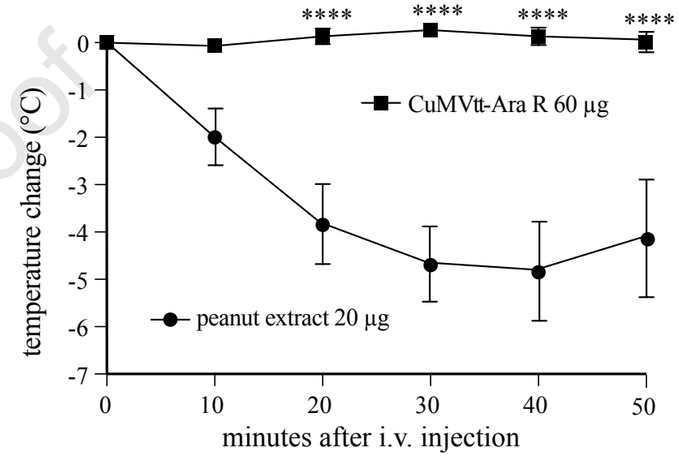

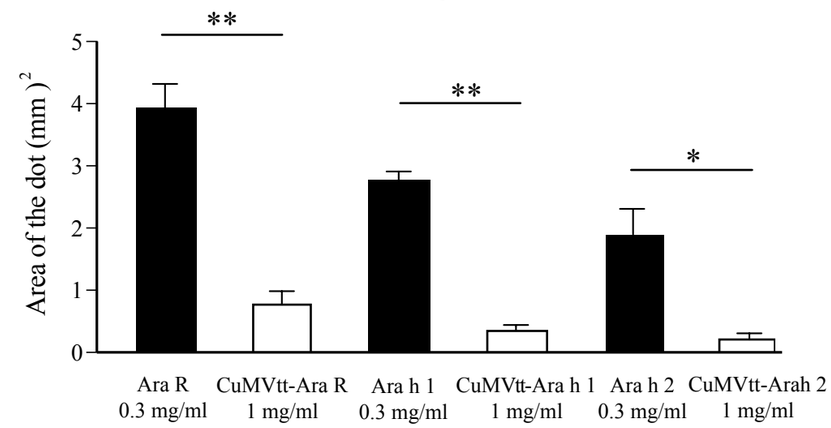

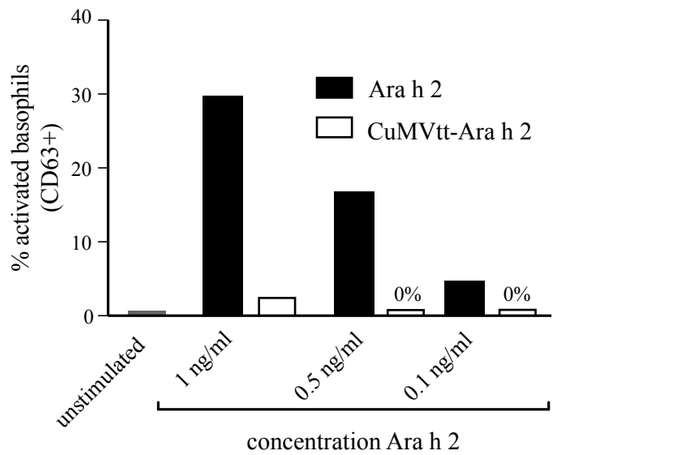

Figure 3

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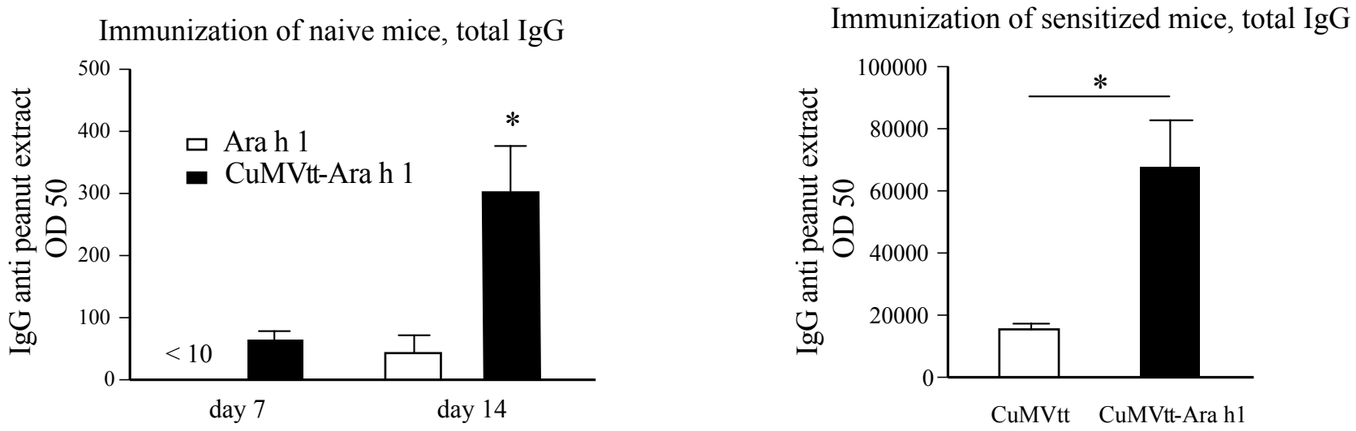
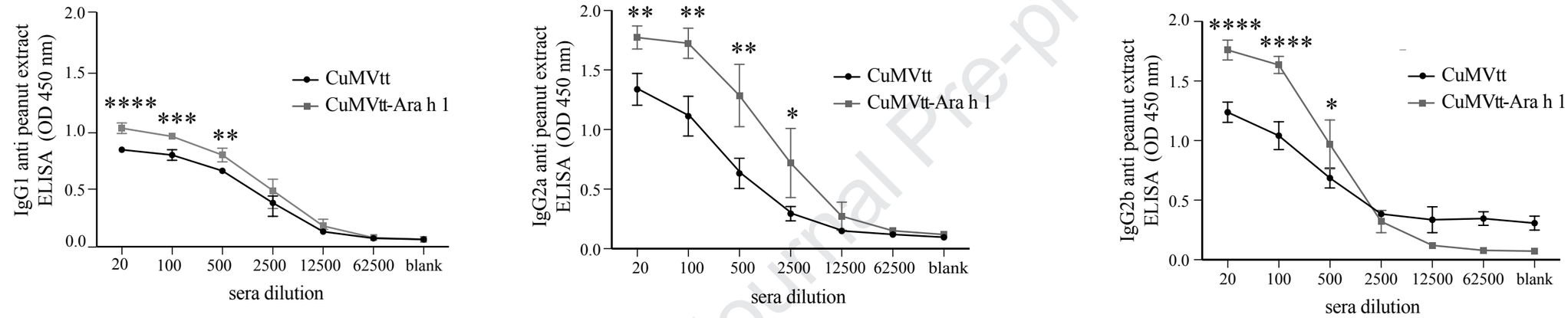
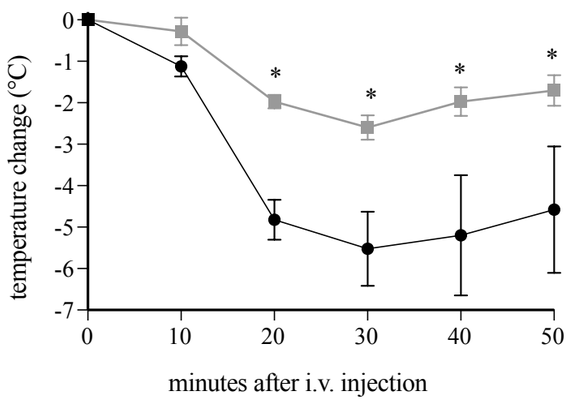


B Immunization of sensitized mice, IgG subclasses



C Passive vaccination



Transfer of 150 µg IgG from naive mice

Transfer of 150 µg IgG from mice vaccinated with CuMVtt-Ara h 1

Figure 4

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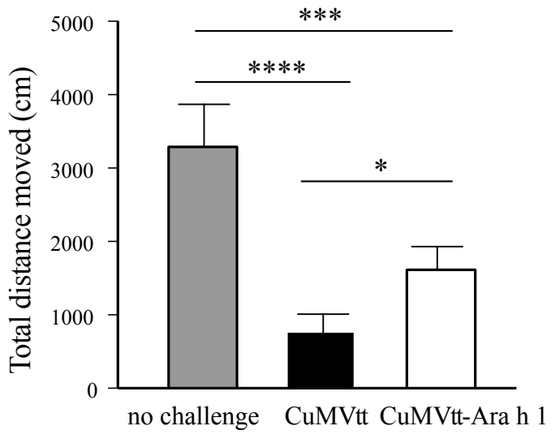

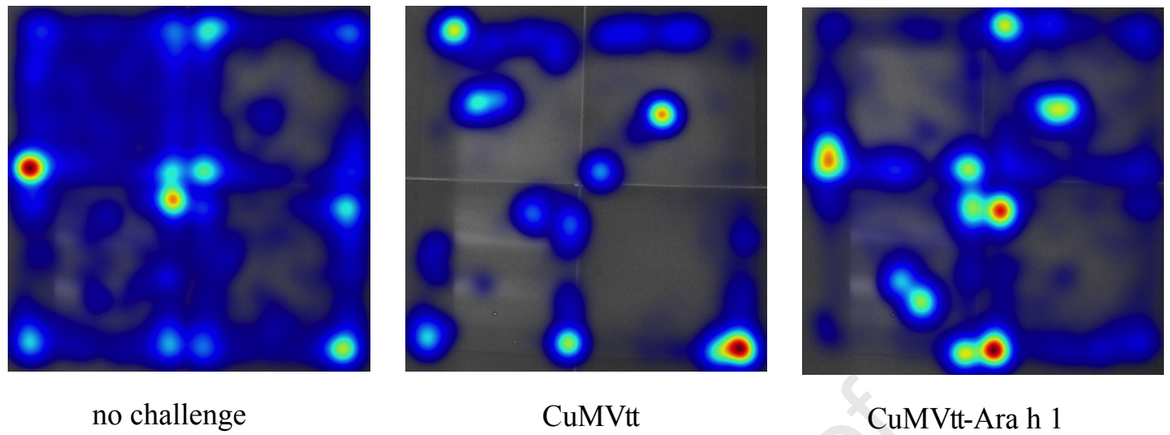

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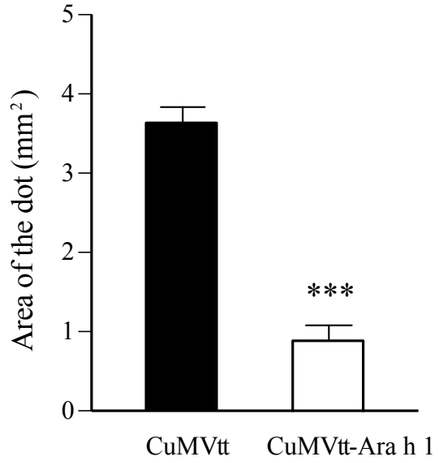

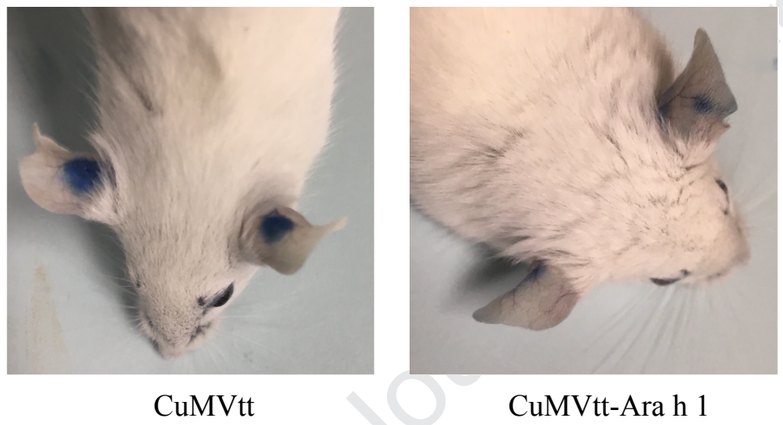

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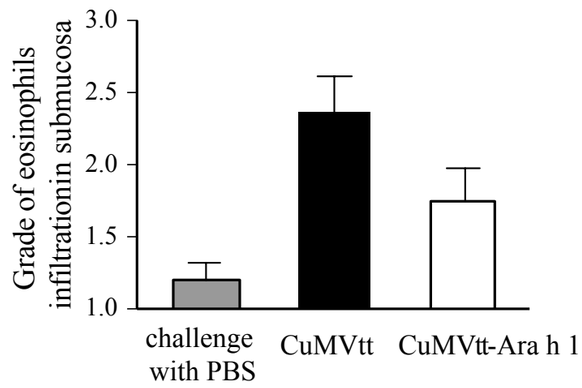

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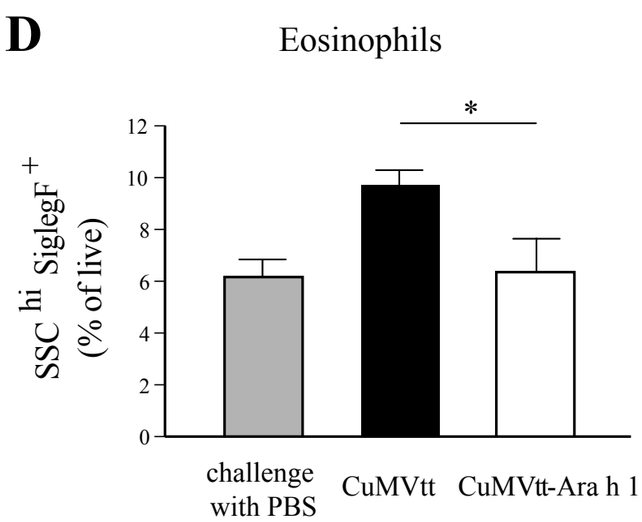

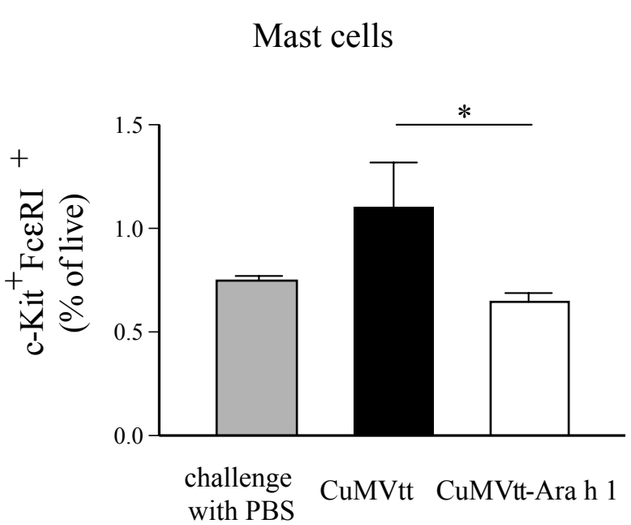

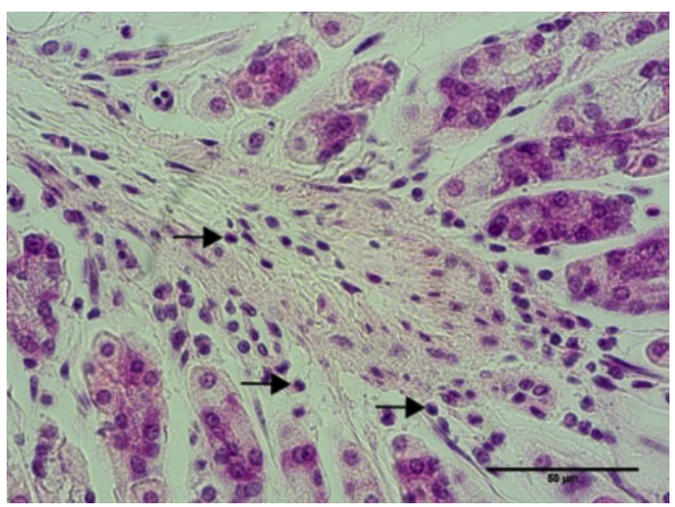

Figure 5

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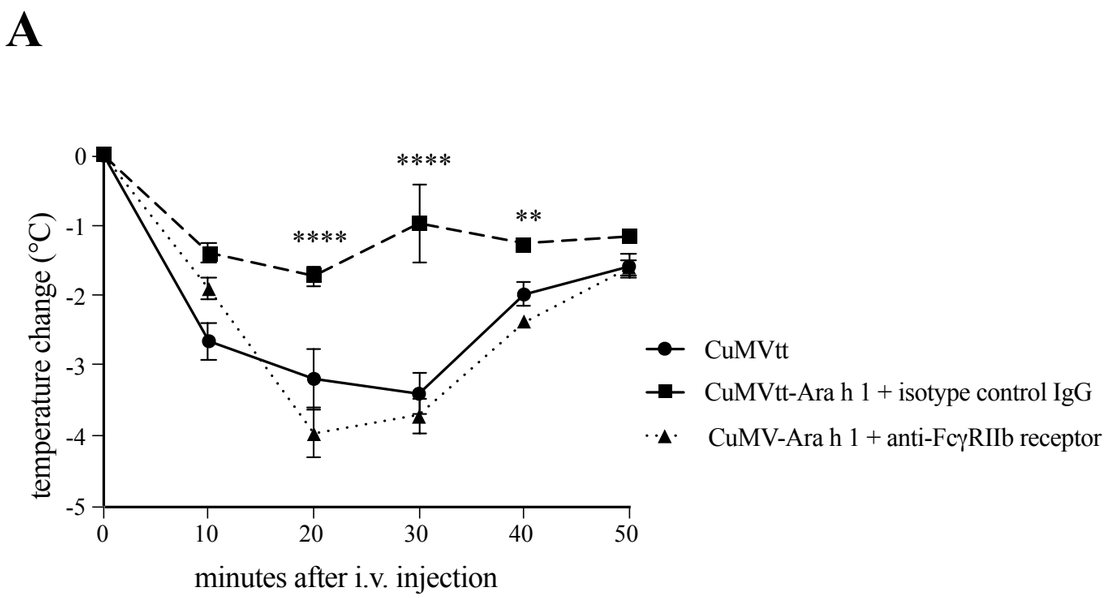

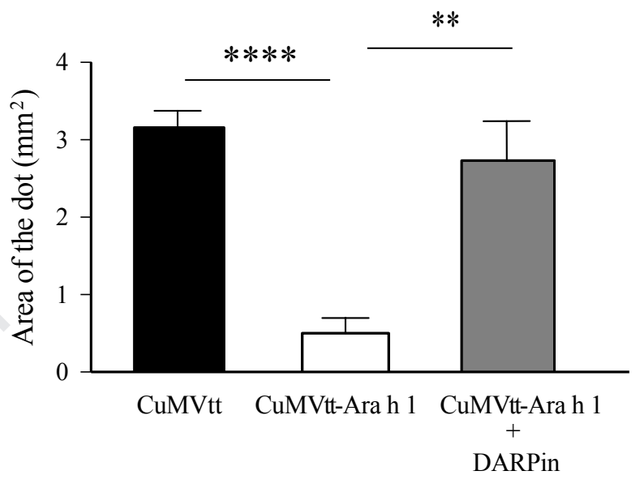

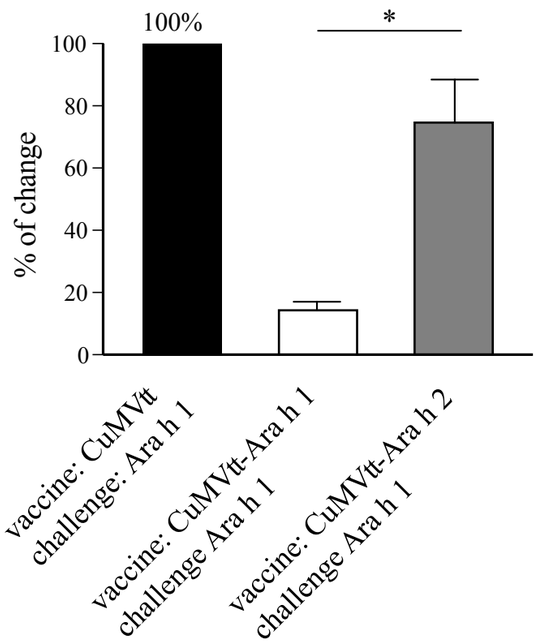

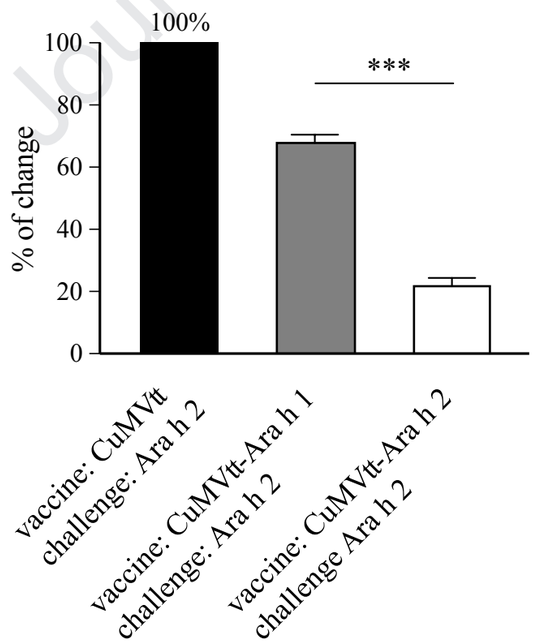

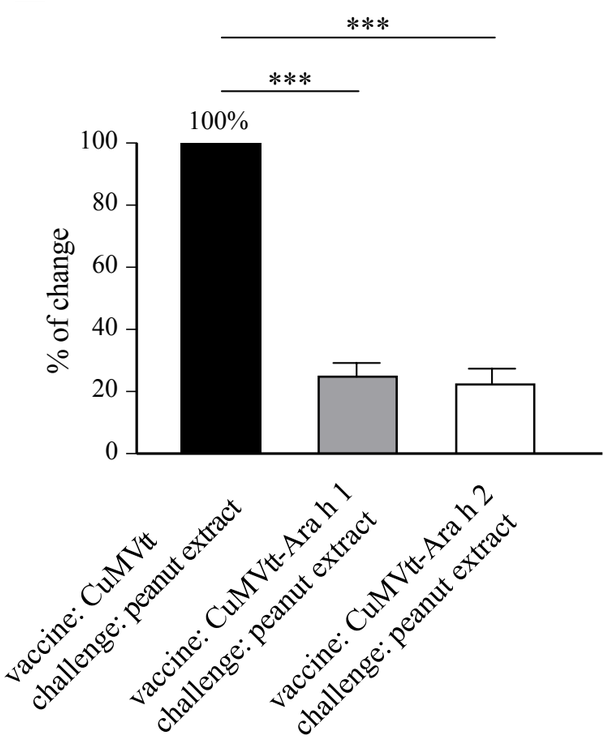

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Figure 6

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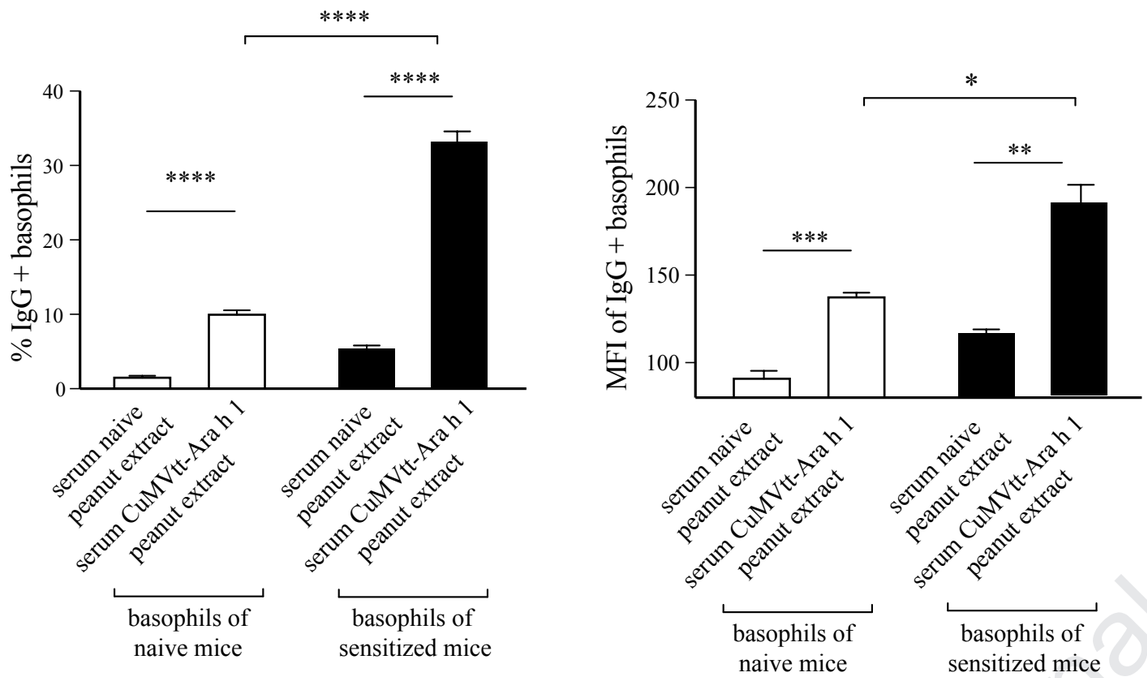
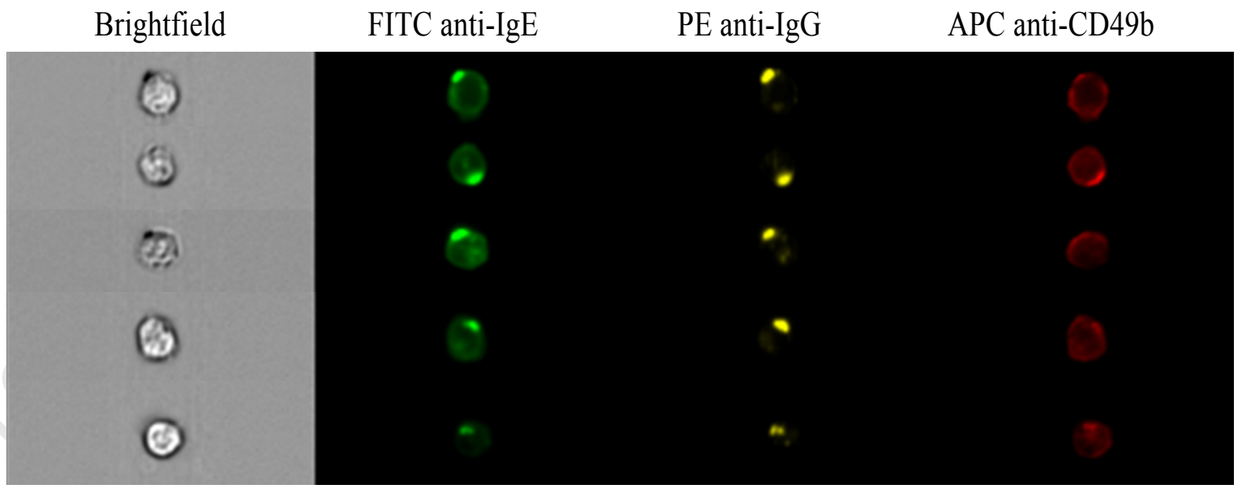


B IgG positive basophils of sensitized mice incubated with serum of mice immunized with CuMV-Ara h 1 and peanut extract



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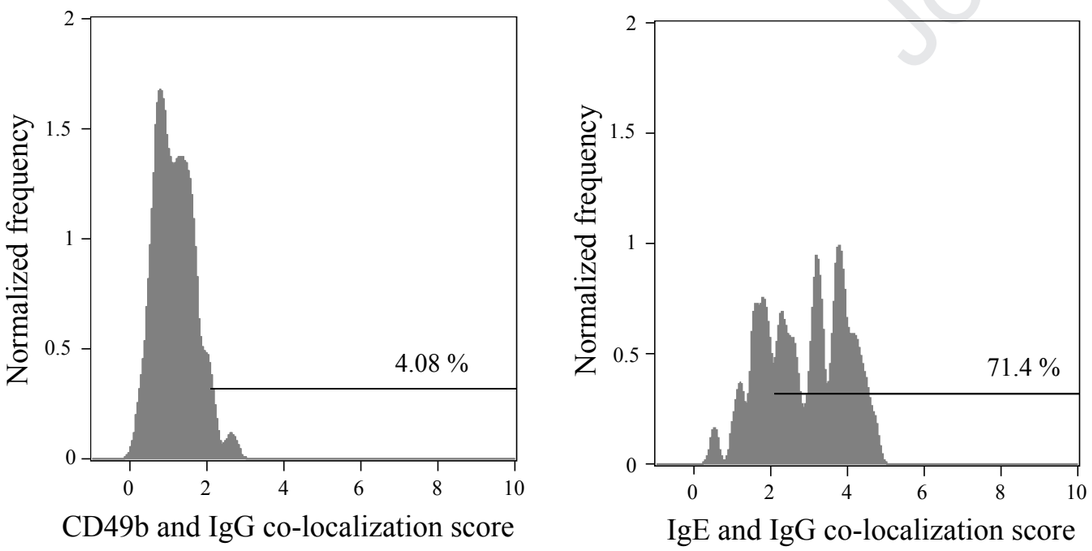

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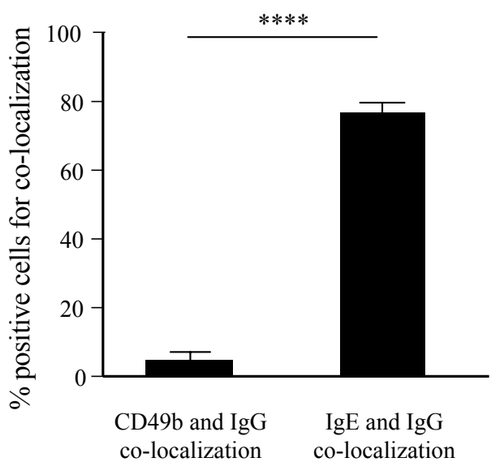

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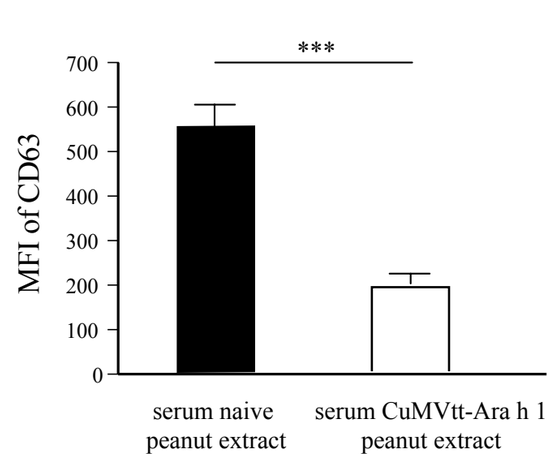

Bone marrow derived mastcells
incubated with serum of sensitized mice

Figure 7

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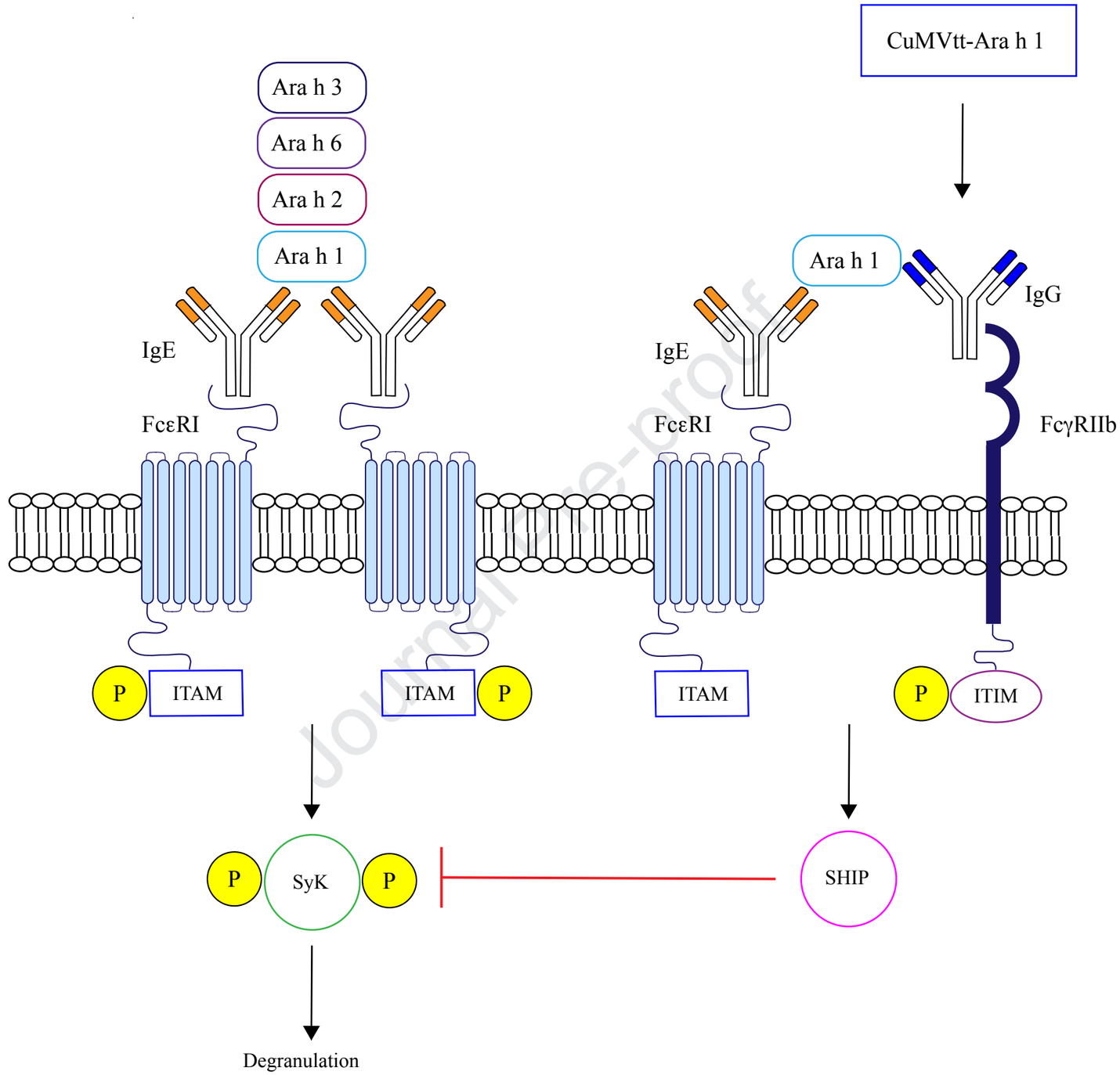

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Legends Supplementary Figures

Supplementary Figure S1.

Characterization of Ara h 1. A) Enrichment of Ara h 1 by ammonium sulfate precipitation (panel I). Ara h 1 can be enriched by ammonium sulfate precipitation, most part of Ara h 1 is soluble. M - protein size marker (Thermo Scientific; #26619); 0 – native peanut extract; AmS - proteins pelleted with corresponding amount (1.3 – 4 M) of ammonium sulfate; Ex - solubilized proteins from AmS pellets. For Western blots, anti Ara h 1 (panel II) pAbs from Indoor Biotechnologies were used as primary antibodies, secondary antibodies - HRP-conjugated antirabbit IgG produced in goat (Sigma). B) Anion-exchange chromatography of Ara h 1 using Sepharose QHP column (panel I) SDS-PAGE analysis of Ara h 1 purification (panel II). M - protein size marker (Thermo Scientific; #26619); S - start material (peanut extract after precipitation with 4M ammonium sulfate); 2 - 11 - Sepharose QHP fractions. C) Size-exclusion chromatography of Ara h 1 using Superdex 200 column (panel I). SDS-PAGE analysis of Ara h 1 purification (panel II). M - protein size marker (Thermo Scientific; #26619); 2 - 11 - Superdex 200 fractions. D) Dynamic light scattering analysis of purified Ara h 1. Ara h 1 solution (1 mg/ml) was analyzed on a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK). The results of three measurements were analyzed by DTS software (Malvern, version 6.32). The average hydrodynamic diameter (Z(av)) was found 18.6 nm.

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Supplementary Figure S2.

Characterization of Ara h 2. A) SDS-PAGE (panel I) and Western blot (panel II) analysis of Arah202 inclusion body solubilisation and refolding. M - Protein size marker (Thermo Scientific Page Ruler Plus, #26619; T – total proteins in recombinant E.coli cells, S – soluble proteins after corresponding treatment, P – insoluble proteins after corresponding treatment. For Western blot, the refolded Arah202 was blotted onto nitrocellulose membrane and treated with Ara h 2 pAbs (Indoor Biotechnologies, produced in rabbits) as primary antibodies; as secondary antibodies HRP-conjugated antirabbit IgG produced in goat (Sigma) were used. B) Anion-exchange chromatography of refolded Arah202 using Sepharose QHP column (panel I). SDS-PAGE analysis of Arah202 purification (panel II). M - protein size marker (Thermo Scientific; #26619); 4 - 12 - Sepharose QHP fractions. C) Size-exclusion chromatography of Arah202 using Superdex 200 column (panel I). SDS-PAGE analysis of Arah202 purification (panel II). M - protein size marker (Thermo Scientific; #26619); 3 - 11 - Superdex 200 fractions. D) Dynamic light scattering analysis of purified Arah202. Arah202 solution (1 mg/ml) was analyzed on a Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK). The results of three measurements were analyzed by DTS software (Malvern, version 6.32). The average hydrodynamic diameter (Z(av)) was found 11.6 nm for most part of the protein. Arah202 partially forms also stable aggregates >100 nm.

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Supplementary Figure S3.

A) Gating strategy for flow cytometric quantification of small intestinal lamina propria eosinophils. Doublets and dead cells were excluded before gating on $CD45^{+}$ $SiglecF^{+}$ SSC^{hi} eosinophils. B) Gating strategy for flow cytometric quantification of small intestinal lamina propria mast cells. Doublets and dead cells were excluded before gating on $CD45^{+}$ $FcR\varepsilon1^{+}$ $cKit^{+}$ mast cells. C) Subcutaneous allergen injection induces anaphylaxis. To investigate the effect of subcutaneous allergen application, mice (n = 3 per group) were injected s.c. with 15 µg dose of free peanut extract (comparable dose of allergen coupled to the CuMVtt used for vaccination). After s.c. allergen injection mice develop anaphylactic clinical signs like immobility and erected hairs and a relevant drop in temperature.

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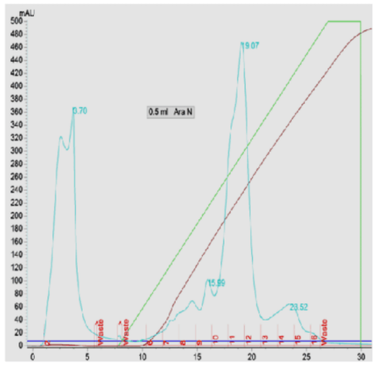
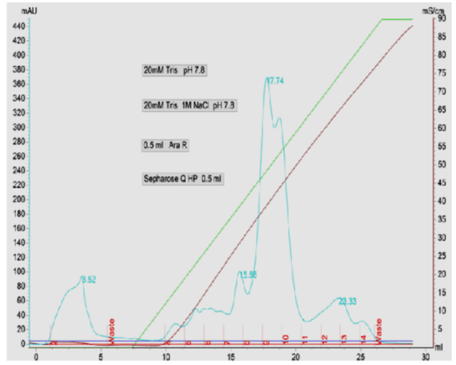
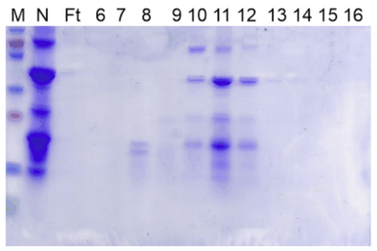
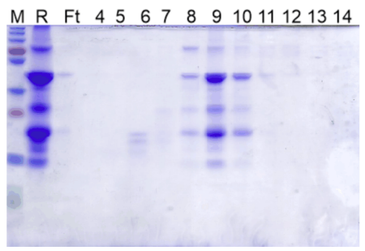
A

Fractionation of peanut extracts
Anion-exchange chromatography (Sepharose Q 0.6 ml column)





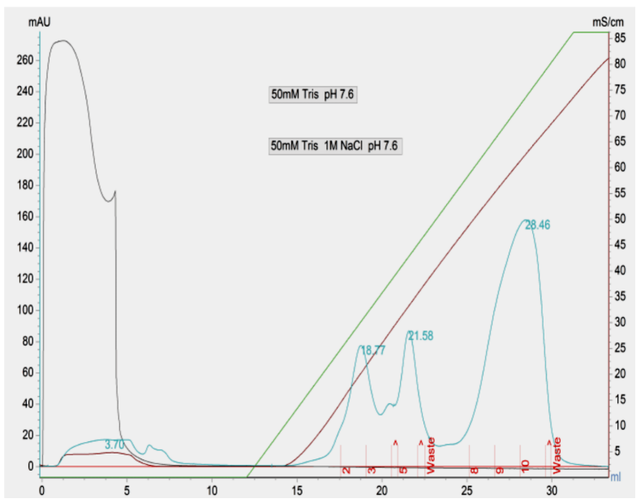


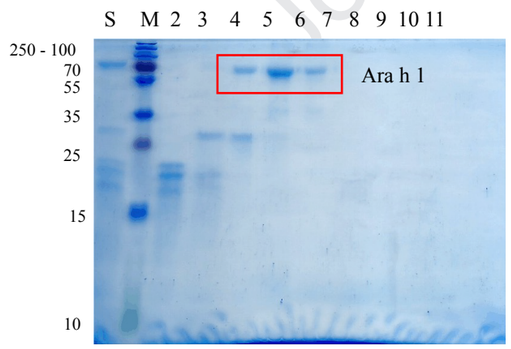


N - extract from native peanuts
R - extract from roasted peanuts
Ft - unbound proteis
4 - 16 - proteins in corrresponding fraction

B

Ara h 1 purification: anion-exchange chromatography

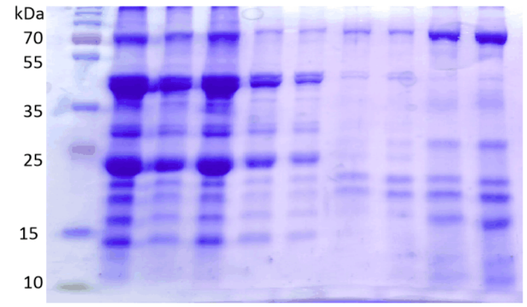




M - protein size marker protein size marker,
PageRuler™ Plus Prestained Protein Ladder 10 to 250 kDa (Thermo Fisher Scientific)
S - start material (native peanut extract 4M AmS extract)
2-11 - Sepharose QHP fractions (0.6 ml column)

C

Ara h 1 purification: analysis of ammonium sulfate precipitations



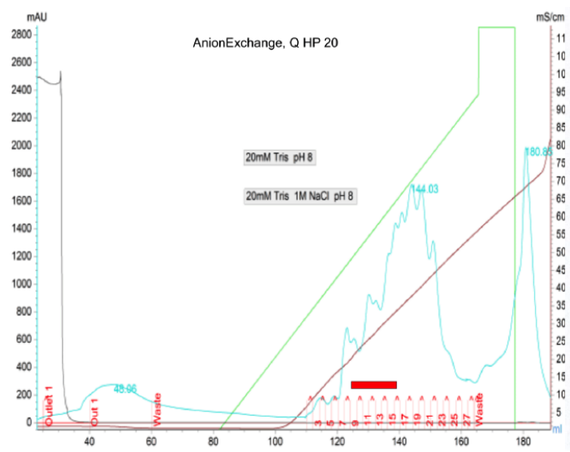
anti-Ara h 1

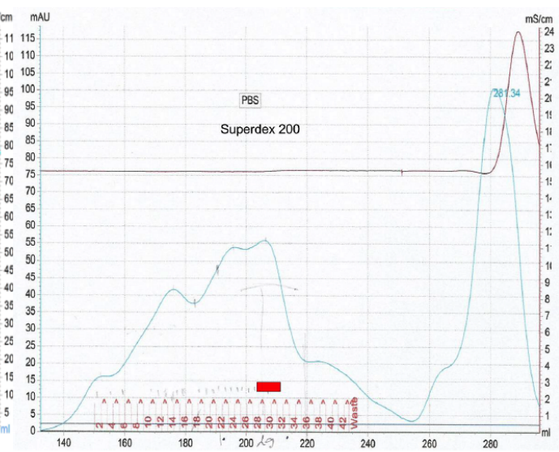
M - protein size marker, PageRuler™ Plus Prestained Protein Ladder 10 to 250 kDa (Thermo Fisher Scientific)
0 - native peanut extract from -70°C
AmS - protein pelleted with corresponding amount of ammonium sulfate
Ex - solubilized proteins from corresponding AmS pellets

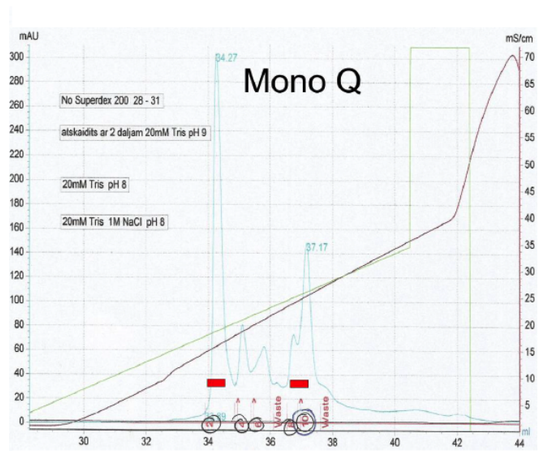
Western blot
primary antibody: anti-Ara h 1 (Indoor Biotechnologies, produced in rabbit),
secundary antibody: HRP-conjugated antirabbit IgG (Sigma, produced in goat)

D

Recombinant peanut allergen Ara h 2 (2.02) purification using Superdex 200 column







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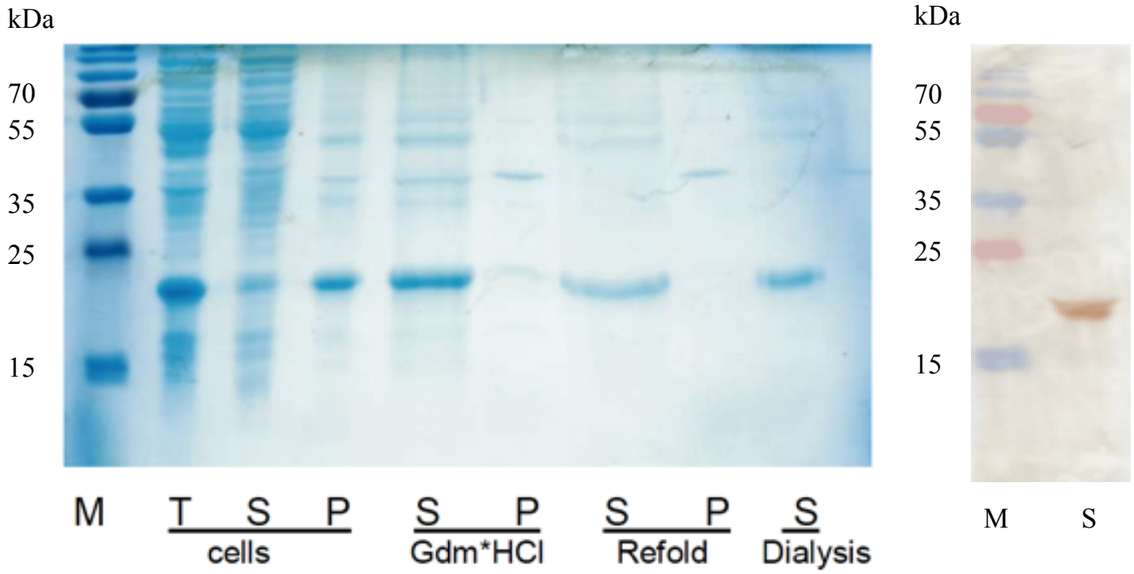
A

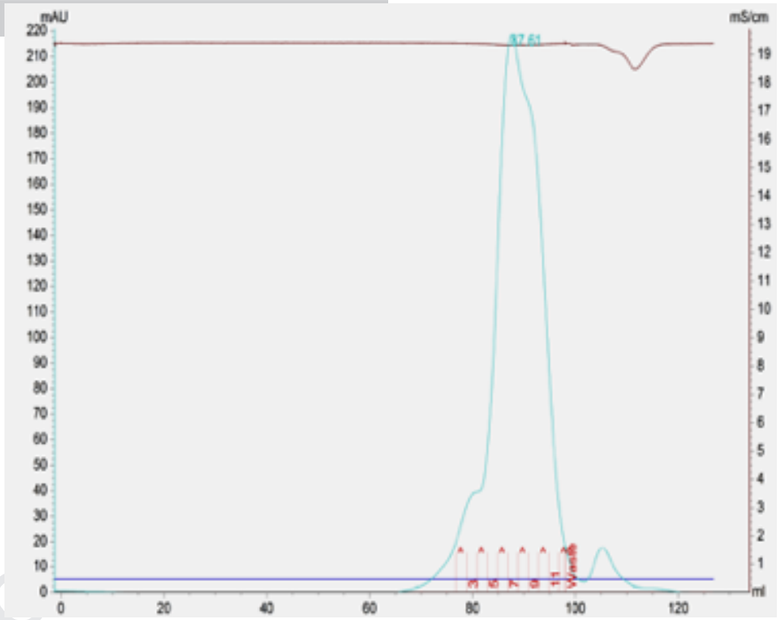
I



II

C

I



B

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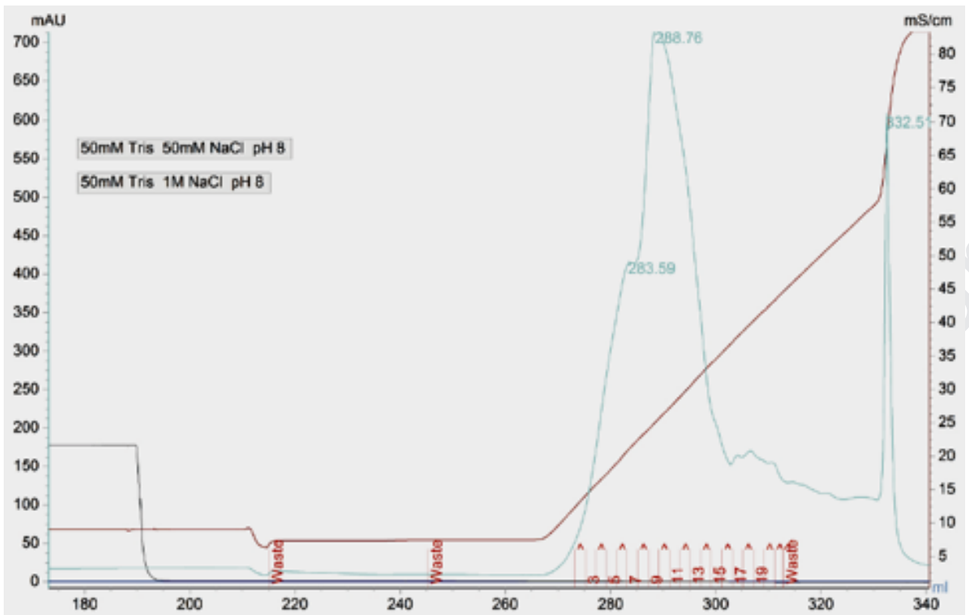

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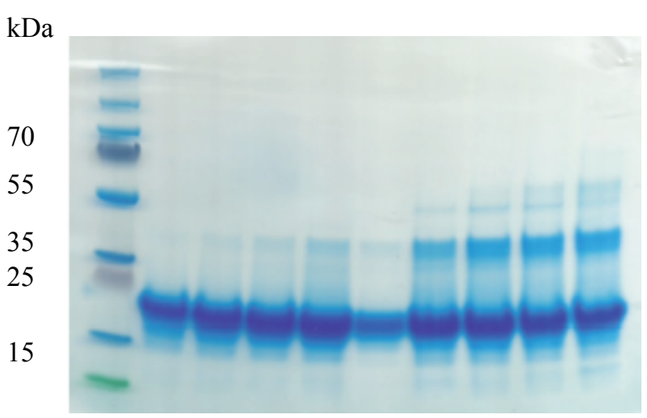

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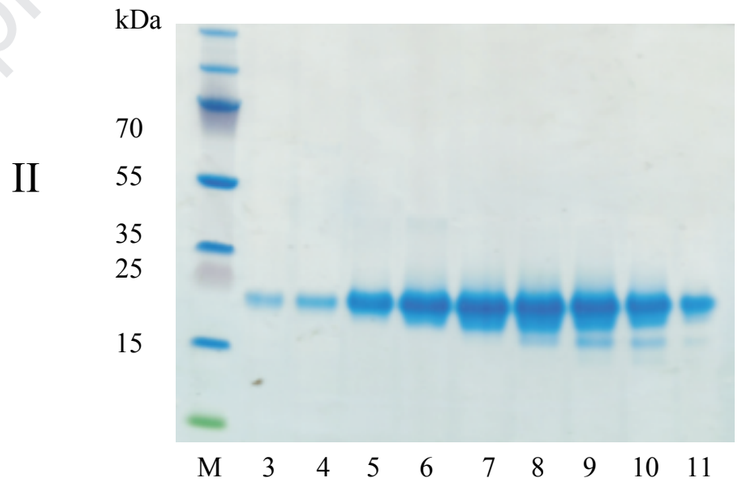

D

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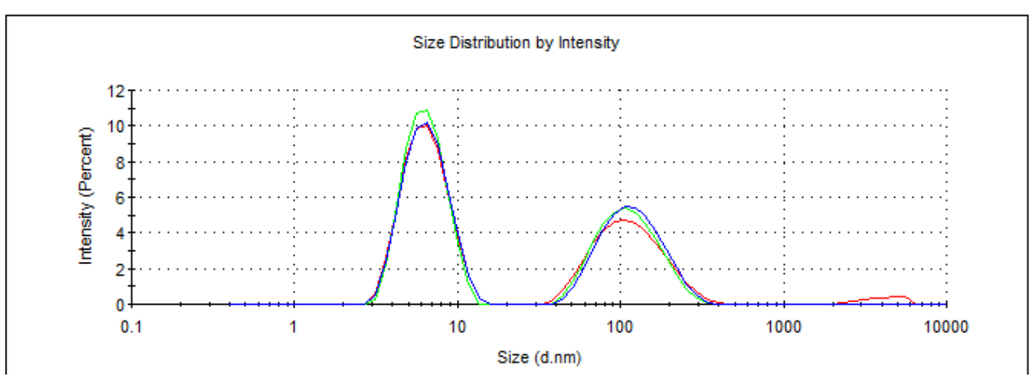
PdI: 0.491	Peak 2:	125.4	42.4	53.29
Intercept: 0.879	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report



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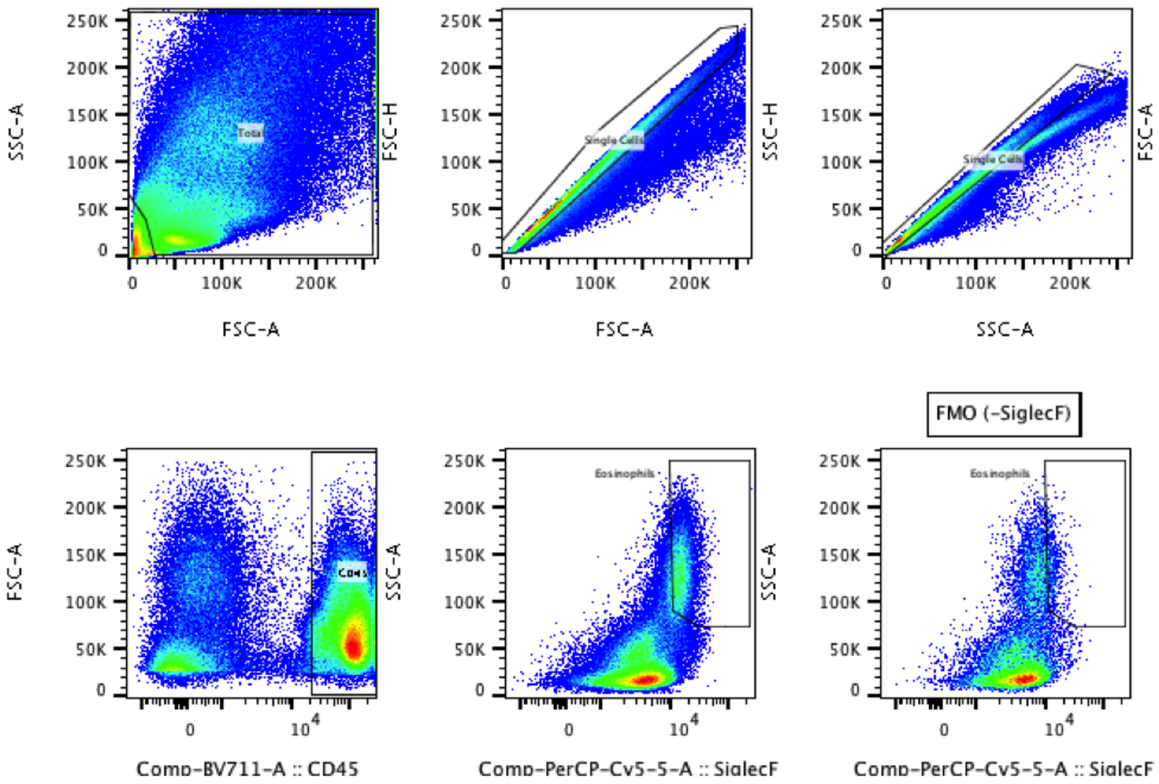
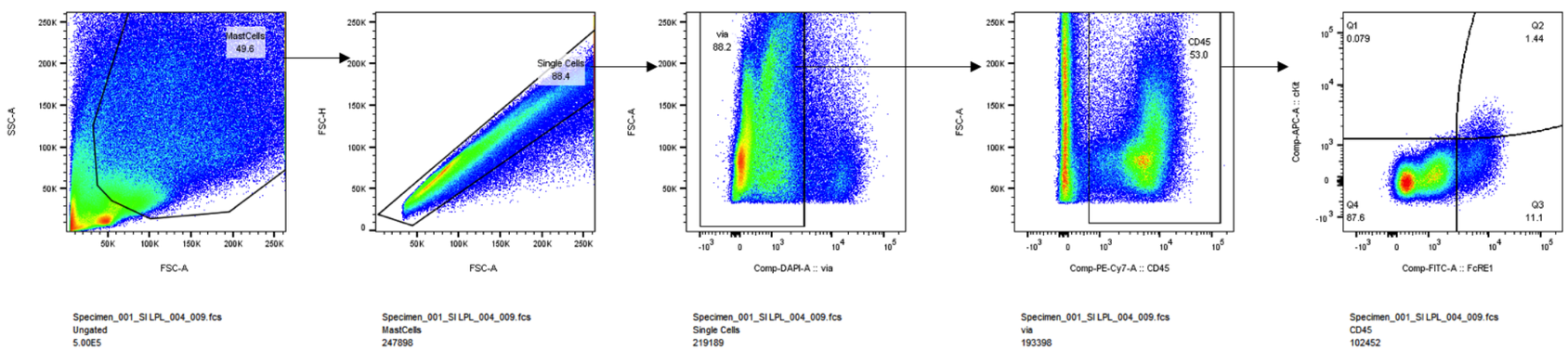
A Gating strategy for Lamina propria eosinophils



B Gating strategy for Lamina propria mast cells



C Subcutaneous injection of peanut extract induces anaphylaxis